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NOVEL APPROACHES FOR VACCINATION AGAINST EQUINE VIRAL ARTERITIS

Javier Castillo-Olivares

A thesis submitted in fulfilment of the requirements of the
Open University for the degree of Doctor of Philosophy

Centre for Preventive Medicine
Animal Health Trust

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"Il n'y a pas de simplicité véritable; il n'y a que des simplifications." ("There is no real simplicity, there are only simplifications.")

Léon-Paul Fargue

Preface

The work reported in this dissertation was carried out in the premises of the Animal Health Trust. The studies represent work carried out by Javier Castillo-Olivares. Where assistance was given due acknowledgement is given in the text. This dissertation has not been submitted as whole or in part to any other university.

To the memory of my Father,

To Sara, Luis Felipe and Roberto

To my mother,

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I would like to thank my two supervisors, Dr. Nick Davis-Poynter and Dr. Duncan Hannant for their encouragement and advice throughout the course of this study. Prompt and detailed reading and scrutiny of manuscripts by Dr Davis-Poynter have been very helpful. Discussions of immunology topics with Dr Hannant have been enjoyable and fruitful. I would also like to thank Dr. Jenny Mumford for her continuous support over the years.

Finally, my friends (in Liverpool and London) and my family, in particular my wife Sara, have constantly been a source of encouragement.

Summary

This thesis explores the potential of two prototype ('marker') vaccines with differential diagnostic capability for improved control of equine viral arteritis. The rationale for using a sub-unit adjuvanted vaccine and a recombinant live virus vaccine, analysis of immune responses they induce and assessment of the protection against infection in experimental conditions is described and discussed in this thesis. Also, the development of new methods for measuring cytotoxic T-lymphocyte responses to EAV is described, increasing the repertoire of immunological assays for EAV infections. Finally, attempts to determine the antigenic specificity of the cytotoxic T-lymphocyte response have been taken.

The sub-unit vaccination approach developed in this project derives from the findings of Dr. Ewan Chirnside *et al* (1995a). This strategy has been developed further. A recombinant protein expressed in *E.coli* comprising the putative ectodomain of the large envelope glycoprotein (G_L) of equine arteritis virus (EAV) was used as a vaccine in ponies resulting in the induction of virus neutralising antibody (VNAb) responses which are comparable in titre to those induced by inactivated whole virus vaccines. The protection afforded by this vaccine against virulent challenge, which correlated with the pre-challenge VNAb titres, was characterised by reduction of nasal virus excretion and viraemia.

A second 'marker' vaccination strategy for EAV was explored. A recombinant live virus containing a deletion of a neutralisation domain on G_L was constructed

by manipulations of the cDNA full length clone of EAV. A synthetic peptide (Peptide-1) whose sequence is contained within the deleted fragment is the basis of an already developed antibody capture diagnostic ELISA test for EAV. The deletion mutant virus (EAV-G_LΔ) showed *in vitro* slightly slower growth kinetics than wild type EAV and when administered by the nasopharyngeal route to ponies these experienced an asymptomatic infection, although virus was recovered from blood and nasal secretions. Both inoculated ponies developed a discriminating antibody response which lacked the Peptide-1 antigen specificity. The serum VNAb response was of high titre against EAV-G_LΔ but of low titre against wild type EAV. Yet, the EAV-ΔG_L inoculated ponies were highly protected against virulent challenge. This novel approach shows great potential as a marker vaccine. Safety and duration of immunity need to be investigated. The structure of VNAb epitopes and effector mechanisms of immunity stimulated by administration of EAV-ΔG_L deserve also attention.

The serum VNAb response that invariably develops in the infected animal lasts for many months or years and is believed to play an important role in virus clearance. However, very little was known about the cellular immunity against EAV due the lack of methods to evaluate these immune responses. Methods to detect cytotoxic T-lymphocyte (CTL) precursors in the peripheral blood of EAV convalescent ponies are described using a ⁵¹Cr release cytolysis assay. Primary equine dermal cells, used as CTL targets, were shown to express MHC-I but not MHC-II, retain ⁵¹Cr efficiently and support EAV replication. Peripheral blood mononuclear cells (PBMC) collected from EAV convalescent ponies that have been incubated with or without live EAV were used as effectors. EAV induced

PBMC cultures showed evidence for expansion and activation of lymphoblasts, with an increase in the CD8+/CD4+ ratio in comparison to mock-induced PBMC. The cytotoxicity induced by EAV stimulated PBMC was virus specific, showed genetic restriction, was mediated by CD8+ T lymphocytes and could be detected for periods between 4 months to more than 1 year post-infection. These findings and methods will hopefully contribute to understanding of virus-host interactions in horses, in particular the mechanisms of virus clearance occurring during EAV infection.

The last part of this project described efforts to try to identify proteins serving as targets for EAV specific CTL's. Target cells transiently expressing EAVORF7 were used in the standard CTL assay developed during the course of these studies (Chapter 4). Cloning of EAVORF7 into the mammalian expression vector pcDNA3 for subsequent expression in different cell lines is described. Whilst, expression of EAV N protein in COS7 and RK-13 cells using plasmid transfection techniques was achieved, EDC's failed to express N efficiently. Unlike plasmid transfection, baculovirus transduction appeared to be a suitable method to achieve protein expression in a high proportion of EDC. The levels of expression of EAV N antigen in transduced EDC, which occurred without the loss of their original morphology, suggested *a priori* that this method was adequate to identify CTL targets. Time and resources constraints limited the number of experiments performed but sufficient progress was made to indicate that this expression technique has good potential for examining EAV CTL targets.

In this research project two novel approaches have been used for the generation of marker vaccines against EAV and methods to evaluate cytotoxic T lymphocyte responses developed. The essential findings of the thesis provide new and interesting information about EAV vaccinology/immunology that now provide the basis for rational approaches to be undertaken to disease prevention.

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Chapter 1. Introduction

Equine viral arteritis is a systemic disease that affects horses, mules, donkeys and zebras worldwide. It was probably first described in the 19th century with the name of 'pink eye' (Clark, 1892) or equine influenza (Pottie, 1888) but the aetiology was unknown until 1953 when the virus was first isolated from an abortion and respiratory disease outbreak in Bucyrus, Ohio (Doll *et al.*, 1957). The virus was then named equine arteritis virus (EAV) for the widespread lesions it caused to the vascular system and since then the disease has been reported frequently in different parts of the world.

1.1. Equine Arteritis Virus (EAV)

EAV is a single stranded RNA enveloped virus initially classified as a member of the family *Togaviridae* (Cavanagh *et al.*, 1994; 1997). However, important advances in the molecular biology of EAV made in the last 15 years revealed that its genome organisation and RNA replication strategies are similar to those of Coronaviruses and Toroviruses and therefore these viruses have been reclassified in the order Nidovirales (Snijder and Meulenberg, 1998). EAV is the prototype of the genus *Arterivirus* of the family *Arteriviridae* that includes also porcine reproductive and respiratory syndrome virus (PRRSV), lactate dehydrogenase elevating virus (LDV) and simian hemorrhagic fever virus (SHFV).

1.1.1. Virus structure

EAV, like other arteriviruses, is an enveloped rounded virus of 50 nm diameter. It consists of an isometric core of approximately 25 nm, formed by a single protein (the nucleocapsid protein N), containing an infectious single stranded positive-sense RNA molecule. The envelope is acquired by budding of already formed nucleocapsids into the smooth endoplasmic reticulum and Golgi followed by release of enveloped virions from the cell by exocytosis (Wood *et al.*, 1970; Magnusson *et al.*, 1970; Wassenaar *et al.*, 1997). A total of six envelope proteins have been characterised for EAV (G_s or GP2b, E, GP3, GP4, G_L or GP5 and M) and PRRSV (E, GP2a, GP3, GP4, G_L or GP5 and M), whereas only three have been characterised for LDV (VP-3M, VP-3P and M/VP-2) and SHFV (p42, p54 and M) (de Vries *et al.*, 1992; 1995a; 1995b; Snijder *et al.*, 1999; Wieringa *et al.*, 2002; Mardassi *et al.*, 1996; Meulenberg *et al.*, 1995b; Meulenberg *et al.*, 1997; van Nieuwstadt *et al.*, 1996; Wu *et al.*, 2001; Faaberg and Plagemann, 1995; Godeny *et al.*, 1995). The large spikes typical of coronaviruses are absent from arteriviruses although ring-like structures (Hylseth *et al.*, 1973), spicules (de Vries *et al.*, 1994) or 'Y' shaped formations (Magnusson *et al.*, 1970) have been observed in EAV virions projecting out of the membrane as determined by morphogenesis studies using electron microscopy. A typical feature of arteriviruses is the formation of double membrane vesicles during the first few hours of infection in the perinuclear region (Breese and McCollum, 1973) where the replicase sub-units that contain the replicase and helicase functions are also localised (van Dinten *et al.*, 1996).

1.1.2. Genome organisation, translation and virus replication

The EAV genome consists of an infectious single stranded RNA molecule of 12704 nucleotides excluding the poly (A') tail. Indeed, Breese and McCollum (1973) were able to incorporate [3H]uridine into virus particles demonstrating the virus contained RNA, and Van der Zeijst (1975) showed that actinomycin D did not inhibit EAV replication and obtained virus after transfection of RNA purified from virions. The genomic RNA of EAV is polyadenylated at the 3' end (van Berlo *et al.*, 1982). At the 5' end, the genomic RNA is believed to have a type I cap structure similar to that described in SHFV (Sagripanti *et al.*, 1986). This structure is thought to be important for infectivity as demonstrated in PRRSV (Meulenber *et al.*, 1998).

The polycistronic arterivirus genome contains two large replicase open reading frames that occupy the 5' three quarters of the RNA and a set of 7 to 10 downstream genes which encode the structural proteins (Snijder *et al.*, 1999; de Vries *et al.*, 1992; Meulenber *et al.*, 1997; Plagemann, 1996). Each ORF has sequences at the 5' and 3' ends that overlap with neighbouring genes. In EAV, ORF's 1a and 1b encode the replicase proteins whereas ORF's 2a, 2b, 3, 4, 5, 6 and 7 encode proteins E, G_s, GP3, GP4, G_L, M and N respectively. The same number of structural genes has been found in PRRSV. The structural nature of LDV ORF's 2a (E), 2b (VP-3M), 5 (VP-3P), 6 (M) and 7 (N) has been demonstrated whereas the function of ORF's 3 and 4 still remain to be determined. Interestingly, the SHFV genome encodes 10 putative structural genes (ORF's 2a, 2b, 3, 4a, 4b, 5, 6, 7, 8 and 9) of which a structural function has only been assigned for ORF's 8 and 9.

The coding regions of the arteriviruses genome are flanked by 5' and 3' non-coding sequences of 156-221 and 59-117 nucleotides respectively. In addition to the aforementioned ORF's, an additional ORF upstream of EAV ORF1a encoding a hypothetical 37 amino acid peptide has been described (Kheyar *et al.*, 1996). Potentially this could be expressed abundantly since it is contained in the leader sequence shared by a set of subgenomic viral m-RNA's. Indeed, apart from the messenger genomic viral RNA, six smaller subgenomic m-RNA's are detected in EAV infected cells (van Berlo *et al.*, 1982; 1986a; 1986b). These hybridised with cDNA copies derived from the smallest of the sub-genomic RNA's, RNA-7, in Northern blots (van Berlo *et al.*, 1986b) indicating that all subgenomic RNA molecules possess common sequences. Corroboration of these findings was made by de Vries *et al.*, (1990) providing evidence as well that these molecules form a nested 3' co-terminal set and contain a common leader sequence of 206 nucleotides at the 5' end which is fused to the body of the m-RNA by the junction sequence 5'UCAAC 3' (Fig. 1.1). This leader sequence derives from the 5' terminal end of the genome. The subgenomic m-RNA's contain the 3' terminal end of the genome and extend for various distances towards the 5' end. Part of the sequence of each subgenomic RNA is contained within the 3' portion of the next larger mRNA. The existence of the nested set of 3'co-terminal sub-genomic RNA's has been described for other arteriviruses and coronaviruses (Kuo *et al.*, 1991; Meulenberg *et al.*, 1993b; Baric *et al.*, 1983 and Spaan *et al.*, 1983) and is what gives the order Nidovirales its name (*nidus*, latin for nest). These subgenomic RNA species are formed during a discontinuous transcription mechanism similar to that of coronaviruses (Baric *et al.*, 1983; Spaan *et al.*, 1983). In this model, transcription is initiated from the common

leader sequence at the 3' end of a negative sense genomic RNA. The junction sequence could pair with any of the complementary junction sequences of the negative RNA strand and extend the leader to complete a subgenomic mRNA. However, an alternative nidovirus transcription model has been put forward by Sawicki and Sawicki (1995) that explains the existence of subgenomic replicative intermediates that are mirror images of the subgenomic mRNA's. In this model, the subgenomic positive RNA strands derive from negative subgenomic RNA strands generated by a discontinuous synthesis of the negative RNA (Fig. 1.1).

All virus proteins are translated from the subgenomic mRNA's except the replicase proteins encoded by ORF1a and ORF1b, which derive from the viral genome. The translation of ORF1b requires a frame shift just before the translation of ORF1a is terminated (den Boon *et al.*, 1991). The result is the generation of ORF1a derived and ORF1ab derived polyproteins of 1727 and 3175 amino acids respectively. These are cleaved by three ORF1a encoded proteases (Snijder *et al.*, 1992; 1995; 1996) yielding eight non-structural proteins from ORF1a (nsp1-8) and four non-structural proteins from ORF1ab (nsp 9-12). The nsp1 and nsp2 are cleaved by internal cysteine autoprotease activities whereas the rest of the cleavage sites are mediated by the serine protease nsp4. The helicase and polymerase functions are associated with nsp 9 and nsp 10 respectively (van Dinten *et al.*, 1999; 1996).

1.1.3. Structural proteins and antigens

1.1.3.1. The nucleocapsid protein N

The small, basic, phosphorylated EAV nucleocapsid protein N is a protein of 12-14 kDa expressed from ORF7 (Zeegers *et al.*, 1976; de Vries *et al.*, 1992). The phosphate group is probably acquired after transport of the EAV N protein to the nucleus immediately after translation (Timjs *et al.*, 2002). The protein is then transported back to the cytoplasm where it is required for virus assembly. Its homologues of PRRSV and LDV are also expressed from ORF7, whereas that of SHFV is derived from ORF9. The N protein is abundantly expressed in EAV infected cells and constitutes approximately 40% of the virion mass. The protein is highly antigenic and N specific antibodies have been found in EAV convalescent horses (Chirnside *et al.*, 1995b; Kheyar *et al.*, 1997; MacLachlan *et al.*, 1998). Similarly PRRSV infected pigs develop antibodies to the PRRSV N protein (Meulenberg *et al.*, 1995a; Rodriguez *et al.*, 1997). It is therefore a good candidate for the development of diagnostic tests. Furthermore, monoclonal antibodies have been produced against this antigen (Chirnside *et al.*, 1988; Weiland *et al.*, 1999).

1.1.3.2. The matrix protein M

The major envelope proteins of EAV are encoded by ORF's 5 and 6. The non-glycosylated M protein, encoded by ORF6, has a molecular weight of 16 kDa and forms disulphide linked homodimers in EAV infected cells and heterodimers with the G_L protein (de Vries *et al.*, 1995; Snijder *et al.*, 2003). It appears that only the heterodimers are incorporated into virus particles. The protein has a short stretch of 18 amino acids thought to be exposed at the virus surface. The M

protein has been shown to be recognised by EAV post-infection equine sera using baculovirus or *E coli* expression systems (MacLachlan *et al.*, 1998; Kheyar *et al.*, 1997). However, to date detailed epitope mapping of this protein has been hampered by the absence of MAb's recognising it, in contrast to the homologous protein of PRRSV (Yang *et al.*, 2000; Magar *et al.*, 1997).

1.1.3.3. The large envelope glycoprotein G_L

The expression product of ORF5, the large envelope glycoprotein of EAV, G_L, is a heterogeneously glycosylated protein of 28-42 kDa which forms heterodimers with the protein M (de Vries *et al.*, 1992; 1995). Its topology has not been resolved conclusively but the large hydrophobic domain is predicted to span the membrane one or three times leaving the amino-terminal end exposed at the surface and the carboxyterminus in the cytoplasm. EAV G_L is the immunodominant viral antigen and its antigenic features have been studied in detail over the last ten years. Balasuriya *et al.*, (1993) demonstrated that MAb's with neutralisation capacity recognised a 29 kDa envelope glycoprotein. Subsequently it was shown that these antibodies bound at a region between aa 99-104 in G_L (Balasuriya *et al.*, 1995a). Likewise, Deregt *et al.* (1994) obtained a panel of G_L specific MAb's, neutralising and non-neutralising, one of which was still reactive to a deletion mutant lacking amino acids 116 to 211. Also, neutralising epitopes mapping to amino acid positions 99 and 100 and 96 and 113 were reported in a separate study (Glaser *et al.*, 1995). Furthermore, a GST-fusion protein and a synthetic peptide comprising aa 55-98 and aa 75-97 respectively reacted with EAV convalescent equine sera and induced neutralising antibodies after intramuscular injection to ponies (Chirnside *et al.*, 1995a).

Pepscan analysis of the G_L protein also demonstrated that the predicted ectodomain of G_L is a major immunodominant antigen when EAV specific horse antiserum recognised strongly three synthetic 15-mer peptides encompassing aa 75-90, 79-94 and 83-98 of G_L (Kondo *et al.*, 1997). More detailed neutralising epitope mapping studies using neutralisation escape mutants, neutralising MAb's and field strains of EAV (Balasuriya *et al.* 1997) identified four regions in the G_L ectodomain that participate in the formation of neutralising epitopes. These localise around amino acids 49, 61, 67-90 and 99-106. It appeared that the peptide 99-104 is a dominant neutralisation epitope. However, mutants containing a deletion encompassing amino acid 66 to 112 could still be neutralised by equine antisera suggesting the presence of other neutralising epitopes not represented by the deleted fragment that can be recognised by the equine immune system.

The EAVG_L homologues of PRRSV and LDV (GP5 and VP-3P) are approximately 60 amino acids shorter than that of EAV. As for EAV, these proteins also interact with the proteins encoded by ORF6, PRRSV M and LDV M/VP-2 (Mardassi *et al.*, 1996; Faaberg *et al.*, 1995); are N-glycosylated at 2 to 4 positions in the case of GP5 of PRRSV and at only 2 positions in the case of LDV VP-3P; and are targetted by post-infection sera. Neutralising epitopes map to the putative N-terminal ectodomain (Ostrowski *et al.*, 2002; Wissink *et al.*, 2003; Li *et al.*, 1998).

It is thought that G_L / M heterodimers are implicated in virus receptor binding and membrane fusion since they are relatively abundant in the viral envelope, all

neutralising monoclonal antibodies bind to the predicted G_L ectodomain and G_L contains a large internal hydrophobic domain. However, chimeric viruses in which the ectodomain of G_L has been swapped by that of LDV and PRRSV or in which the ectodomain of PRRSV M was substituted by that of EAV or LDV retained their infectivity to BHK-21 and porcine alveolar macrophages respectively (Dobbe *et al.*, 2001; Verheije *et al.*, 2002), suggesting G_L and M are not the main cell tropism determinants.

1.1.3.4. Other envelope glycoproteins

Apart from the two major envelope glycoproteins an additional four have been identified for EAV as well as for PRRSV. The approximately 10 kDa non-glycosylated E protein of EAV is encoded by ORF 2a (Snijder *et al.*, 1999). Its hydropathy profile suggests is an integral membrane protein with an uncleaved signal-anchor sequence in the central part of the molecule. Its topology has not been elucidated and it could span the membrane one, two or three times. Nothing its currently known about the immunogenicity of this protein. Its homologues in LDV, PRRSV and SHFV are encoded by ORF's 2a, 2b and 4a respectively.

The ORF 2b of EAV encodes the G_S protein, a class I integral membrane glycosylated protein of 25 kDa which is present in virions as a sialylated disulphide-bonded homodimer (de Vries *et al.*, 1995). The protein is poorly immunogenic and very rarely recognised in immunoassays by equine post-infection sera.

More recently (Wieringa *et al.*, 2002) it has been demonstrated that the products of ORF's 3 and 4 of EAV encode structural proteins, although the data available

suggests that they represent a very small percentage of the virion mass. GP3 is a heavily glycosylated protein in which the signal sequence has not been cleaved and that can adopt a type II or type III configuration, being anchored by its signal sequence or at both termini respectively. The EAVGP3 protein is antigenic (Hedges *et al.*, 1999) like its homologues of other arteriviruses (Faaberg *et al.*, 1997; Katz *et al.*, 1995). GP4 of EAV is a type I membrane glycoprotein of 28 kDa which is glycosylated inefficiently although it appears that only the fully glycosylated form of the protein is incorporated into virions. The protein has not been proven to be antigenic for EAV although for PRRSV, antibodies with neutralising capacity react with PRRSV GP4 (Meulenberg *et al.*, 1995b; van Nieuwstadt *et al.*, 1996).

1.1.4 Biological properties of EAV.

EAV infectivity is retained at -70°C for years, at 4°C for > 75 days, at 37°C for 2 or 3 days and at 56°C for just 20-30 minutes (McCollum, 1961). Low pH, ether, chloroform, sodium deoxycholate and phospholipase C inactivate EAV infectivity rapidly, as expected for an enveloped virus. Detergents such as Triton-X-100 (Zeegers *et al.*, 1976) and Nonidet NP-40 (Horzinek *et al.*, 1971), and UV light also inactivate the virus efficiently (de Vries *et al.*, 1996).

Monocytes, macrophages and vascular endothelial cells are the primary target cells of EAV *in vivo* and arteriviruses in general. However, while EAV can grow *in vitro* in a wide variety of cell lines, the cell tropism of other arteriviruses seems much more restricted (de Vries *et al.*, 1996). Thus, EAV has been successfully grown in baby hamster kidney cells (BHK-21) (Hyllseth, 1969),

African green monkey kidney cells (BSC-1) (Crawford *et al.*, 1973), simian virus 40-transformed equine ovary cells (EO), hamster lung (HmLu) (Konishi *et al.*, 1975), canine hepatitis virus-transformed hamster tumour (HS and HT-7) (Shinagawa *et al.*, 1976), cynomolgus monkey kidney (JINET) (Konishi *et al.*, 1975), rhesus monkey kidney (LLC-MK2) (Radwan and Burger, 1973a; b), equine dermal cells (NBL-6) (Breese and McCollum, 1970), rabbit kidney (RK-13) (McCollum, 1970) African green monkey kidney (VERO C1800) (Konishi *et al.*, 1975; de Vries *et al.*, 1993), primary equine embryonic lung (EEL) (Castillo-Olivares *et al.*, 2001). LDV grows in primary cultures of mouse macrophages, but other macrophage cell lines failed to support its replication (Plagemann and Moenig, 1992) and PRRSV and SHFV can only replicate in porcine alveolar macrophages and peritoneal macrophages respectively as well as in cell lines derived from African green monkey kidney such as MA-104, Marc-145, and CL-2621 (Benfield *et al.*, 1992; Kim *et al.*, 1993 and Gravell *et al.*, 1986a; b).

EAV infected cells present cytoplasmic vacuoles containing virus particles (Breese and McCollum, 1970) and they round up and detach from the tissue culture vessel surface allowing easy titration of virus stocks by end-point dilution assays (Hyllseth, 1969; van Berlo *et al.*, 1980; Senne *et al.*, 1985).

1.2. Epidemiology of Equine Viral Arteritis

Various serological studies reveal that EAV has a worldwide distribution including reports from various parts of Europe (Akashi *et al.*, 1976; Burki, 1992; Ceccarelli *et al.*, 1972; Chirnside *et al.*, 1995; Wood *et al.*, 1995 de Boer *et al.*, 1979; Eichorn *et al.*, 1995; Gerber, 1978; Hyllseth and Petterson, 1970;

Klingeborn *et al.*, 1991; McCollum, 1973; McGuire, 1974; Moraillon and Moraillon, 1978; Newton *et al.*, 1999; Weiss *et al.*, 1994; Wood *et al.*, 1995), America (Hullinger *et al.*, 2001; Lang and Mitchell, 1984; McCollum, 1973 ; McCue *et al.*, 1991; McGuire, 1974), Asia (McKenzie, 1988; Akashi *et al.*, 1976; Fukunaga *et al.*, 1994), Africa (Himeur *et al.*, 1975; Moraillon *et al.*, 1978; Paweska *et al.*, 1994) and Australia (Huntington *et al.*, 1990a; b; McKenzie, 1988; McKenzie, 1989; McKenzie, 1990). But the prevalence is variable between different countries and horse breeds. The ubiquitous distribution of the virus contrasts with the relatively few occasions that EAV related disease is reported. Indeed, it has been observed that asymptomatic infections are very common and on many instances the disease passes unnoticed, although reports of abortion and clinical arteritis or foal deaths due to EAV occurred in various outbreaks reported from Austria (Nowotny *et al.*, 1992), Canada (Klavano *et al.*, 1987), Italy (Redaelli *et al.*, 1980), Poland (Golnik *et al.*, 1981, 1986) and Switzerland (Burki and Gerber *et al.*, 1966). These have also been described in the USA such as those reported by Collins (1987) in a teaching veterinary hospital or those described in a trotting race track in Kentucky (McCollum and Swerczek, 1978), or in farms at Washington and Virginia in 1997 (Balasuriya *et al.*, 1998; 1999a) and Pennsylvania in 1996 (McCollum 1999a). Clinical signs were observed too in riding schools in the Netherlands (van Gorkum *et al.*, 1994) and Spain (Monreal *et al.*, 1995), and in stud farms in Germany (Eichorn *et al.*, 1995), Britain (Wood *et al.*, 1995) and France (Zientara *et al.*, 1995).

Susceptible equines acquire the infection via the respiratory route or venereally through infected semen from persistently infected stallions. Indeed, experimental

infections and field studies have shown that aerosol transmission from respiratory secretions of acutely infected animals constitutes a means of spreading the infection to a large number of animals (Doll *et al.*, 1957; McCollum and Swerczek, 1978; McCollum *et al.*, 1971; Cole *et al.*, 1986), but the efficiency of this mode of transmission varies between various epizootics. Epidemiological and experimental data indicate that persistently infected stallions play a central role in the epidemiological cycle of equine viral arteritis. These animals excrete virus in their semen for months or years without displaying any symptoms (Timoney, 1987) and can introduce inadvertently the infection into naïve equine populations after mating to susceptible mares, which become infected by this route with an efficiency of 85-100 % (McCollum *et al.*, 1987; Timoney *et al.*, 1987a, 1987b). Therefore, EAV can be easily introduced into stud farms via infected semen with subsequent horizontal spread by the respiratory route. Artificial insemination with fresh, frozen or extended-frozen semen from persistent shedding stallions has been proven as effective as natural mating for transmission of EAV infection and has been documented as the origin of various outbreaks of EAV (Timoney, 2002). Additionally, EAV can be transmitted from urine (Timoney and McCollum, 1993; Neu *et al.*, 1988; Fukunaga *et al.*, 1981) and other secretions and body fluids such as placenta and amniotic fluid from aborted fetuses (Cole *et al.*, 1986). Other minor sources of infection are through contaminated personnel and fomites (Timoney and McCollum, 1993).

1.3. Pathogenesis

EAV, like all the other arteriviruses, infects primarily macrophage / monocytes and disseminates throughout the organism via these infected cells and probably also as a cell-free virus. The progression of the infection in the horse has been studied by analysis of distribution of viral antigen and lesions in horses sacrificed at different times after challenge with the Bucyrus strain and in natural infections (Del Piero, 2000; MacLachlan *et al*, 1996; McCollum, 1971; Prickett, 1973). These studies showed that the virus first replicates in the respiratory epithelium and alveolar macrophages around 24 hours post-infection and then disseminates to regional lymph nodes by day 3. From there systemic distribution follows via infected macrophages and monocytes and probably cell-free virus with viral antigen present in macrophages and dendrite-like cells of lymph nodes. By day 6 post-infection the virus localizes to the endothelium and medial myocytes of blood vessels and mesothelium. Maximum vascular damage occurs by day 10 post-infection and from this moment virus begins to disappear from all locations except the renal tubular epithelium where the virus can persist for another 2 weeks. Normally, virus is cleared from most sites by day 28 post-infection. However, in an important proportion of stallions (30%-60%) the virus establishes a persistent infection that is localised to the genito-urinary tract (Neu *et al.*, 1988; Timoney, 2002).

The pathogenicity of EAV seems to be exerted via damage of the vascular endothelium leading to alterations in the permeability of the blood vessels causing edema or in more severe cases hemorrhage. The pathogenicity is believed to be dependent on host factors (yet unidentified), environmental

conditions and virus strains. In this respect, McCollum and Timoney (1999b) noted variations in virulence of different EAV isolates according to the severity of clinical signs and lesions they produce after infection, with the velogenic Buc-53 strain being the most pathogenic virus encountered so far. Moore *et al.* (2002) correlated the virulence of different EAV strains in horses with the virus growth characteristics in primary equine endothelial cells. Lymphopenia is a common denominator of most EAV infections, although this seems to be an indirect effect of the infection since lymphocytes have not been identified yet as virus targets. The pathological consequences of monocyte / macrophage infection and effects of EAV in the immune system have not been studied in detail. Arteriviruses cause a cytotoxic effect which occurs by apoptosis and / or necrosis. Apoptosis during PRRSV infection, which occurs in the lymphoid tissues and testicular germ cells, has been suggested as an explanation for the lymphopenia observed in infected pigs (Sirinarumitr *et al.*, 1998). Although the clinical and pathologic significance of apoptotic cell death due to EAV infection (Archambault *et al.*, 1999) remains to be determined, it could explain also the EAV induced lymphopenia in the infected equine.

The pathologic mechanism of abortion has not been clearly identified but it is speculated that it occurs due to a myometritis (Del Piero, 2000). Reduction of blood supply to the foetus due to alterations of vascular tone by inflammatory mediators and compression of blood vessels by edema, reduction of progesterone production by placenta and release of prostaglandins may all contribute to chorionic detachment and foetus expulsion.

1.4. Persistence

A hallmark of arteriviruses biology is the capacity to establish persistent infections in the host (Timoney, 2002; Snijder and Meulenberg 1998, Gravell *et al.*, 1986a; b Plagemann 1996). In horses, EAV persistent infections have only been reported in the stallion, a condition that apparently is dependent on testosterone. Indeed, naturally infected persistently shedding stallions stopped excreting virus in semen after castration, but this can be maintained by administration of testosterone (Little *et al.*, 1992). Likewise, efforts to establish persistent infections in colts infected before puberty have been unsuccessful (Holyoak *et al.*, 1993). EAV has been isolated from vaginal swabs of mares but only during the acute phase of infection (Fukunaga *et al.*, 1981). EAV appears to localise to cells of organs of the stallion urogenital tract during persistent EAV infections. Thus, virus has been recovered from the epididymis, vas deferens, ampulla, seminal vesicle, bulbourethral gland, prostate, urinary bladder and proximal urethra in persistently infected stallions necropsied between days 92 and 148 post-infection (Neu *et al.*, 1988). The mechanisms by which testosterone promotes virus persistence are not known but it is hypothesised that the immunological barriers operating in the male genital tract, both physical and functional, which are androgen dependent, prevent infection being cleared by effector cells of the immune system. Also, testosterone maintains accessory sex glands functional and active, with cells able to support virus replication. These organs become atrophic after castration (Thompson *et al.*, 1980).

An important aspect of EAV persistence is the behaviour of the virus as a quasispecies with emergence of genotypic and phenotypic variants during

persistent infection of stallions (Hedges *et al.*, 1999). New variants could be more adapted to replicate in the respiratory tract and spread rapidly by this route causing outbreaks as was suggested by Balasuriya *et al.* (1999a). The generation of genotypic variants has been documented also for SHFV and LDV (Plagemann, 1996).

Alternative anatomic sites of virus persistence may exist since virus has been isolated sporadically from blood buffy coat samples collected on days 111 and 58 after infection from EAV infected stallions (Neu *et al.*, 1988) and up to day 53 post-infection in geldings (McCollum *et al.*, 1994).

Other arteriviruses also establish persistent infections in their hosts. PRRSV persistent infection in pigs is not restricted to males. Indeed, this virus replicates in testicular germ cells of boars and virus is excreted in the semen of these animals but virus can be detected in cell culture or by RT-PCR in infected pigs, including females, from oropharyngeal scrappings and tonsils (Wills *et al.*, 1997; 2003) up to day 251 after infection. Mice infected with LDV invariably develop a life long persistent infection (Plageman, 1996). The virus infects a sub-population of macrophages that is continually renewed in the liver, spleen, lymph nodes and skin of the host. Also, viral RNA has been detected in germinal centres of lymph nodes and spleen and is associated with polyclonal activation of B cells (Anderson *et al.*, 1995). SHFV causes acute or asymptomatic persistent infections in patas monkeys in which the virus can be detected in the blood of their hosts for long periods of time (Gravell *et al.*, 1986a; b).

1.5. Pathology

Gross and histological lesions of experimental (Holyoak *et al.*, 1993; Prickett *et al.*, 1973; McCollum, 1981; MacLachlan *et al.*, 1996) and natural infections with EAV (Del Piero, 1997; Lopez *et al.*, 1996; Prickett *et al.*, 1973; Vaala *et al.*, 1992) have been reviewed recently (Del Piero, 2000). As a result of the damage to the vascular system, EAV acute infection causes, depending on the virulence of the isolate, generalised edema, congestion and / or hemorrhage of subcutaneous tissues, lymph nodes and various organs including the presentation of a yellowish exudate in pleural and peritoneal cavities. Aborted fetuses can be expelled without premonitory signs and may be either autolysed or well preserved.

Microscopic lesions occur primarily in small arteries and venules and lymphatics of almost all organs, ranging from perivascular edema with a discrete lymphocytic infiltrate and endothelial cell hypertrophy to, in more severe cases, loss of endothelium, fibrinoid necrosis of the tunica media accompanied by an abundant inflammatory infiltrate in the perivascular space and presence of thrombi in the lumen of capillaries. The virus antigen is present in blood vessels in endothelial cells, myocytes, pericytes and endothelium associated macrophages. In the lungs, EAV infection may cause an interstitial pneumonia characterised by macrophage infiltrate and hyperplasia or hypertrophy of pneumocytes with virus antigen present in both these cell types. EAV infected lymphoid tissues present necrosis, edema and slight hemorrhage with histiocytic erythrophagocytosis with viral antigen in macrophages and stromal dendrite-like cells. The intestine may present distension of submucosal lymphatics of the large

intestine with necrosis of the lamina propria. EAV antigen is present in enterocytes. Kidneys may be severely affected presenting tubular necrosis, lymphocytic interstitial nephritis, glomerular tuft disorganisation and hypercellularity. Viral antigen may localize to tubular epithelial cells, glomerular endothelium and mesangial cells. Skin may be affected as well, presenting dermatitis and thrombosis associated with vasculitis. The central nervous system is rarely affected.

In the aborted foetus the lesions are normally mild and are only detected occasionally being perivascular lymphocytic infiltrate and interstitial pneumonia. Antigen can be localised in allantochorion, thymus epithelium, splenic reticular cells, endothelial cells of blood vessels and enterocytes.

1.6. Clinical signs

Symptoms of equine viral arteritis vary widely in severity and range of symptoms displayed by affected animals. This variability depends on the virus strain, environmental factors, age, breed and immune status of the host (Timoney and McCollum, 1993). Thus, infected horses may or may not present any combination of the following clinical signs: fever (which may last up to 6 or 7 days and reach up to 41°C); depression; lethargy; anorexia; weakness; congestion or petechiation of mucosal membranes; palpebral, periorbital or supraorbital edema; conjunctivitis; edema of mammary glands, prepuce, ventral abdomen and legs; urticarial skin rash; ataxia; respiratory distress; rhinitis with serous nasal discharge; diarrhoea and abortion in the pregnant mare. The range of clinical signs vary between outbreaks and even between individuals in the same outbreak. The breed appears to be an important factor in determining what

symptoms can be displayed by the infected animal. It is worth noting that although susceptibility to infection is the same for Standardbreds and Thoroughbreds, clinical disease is less frequently reported in the former, which is an interesting fact considering the high sero-prevalence in this breed. Serosurveys in US and Australia for example demonstrated that 60-90% of Standardbred stallions and mares are positive compared to only 2-3% of Thoroughbreds (McCue *et al.*, 1991; Huntington *et al.*, 1990a; b). Furthermore, experimental infection in donkeys with a moderately virulent strain such as Kentucky-84 failed to induce significant clinical signs (McCollum *et al.*, 1995) suggesting the genetic background of the host might play a role in pathogenicity. The virus strain is a factor determining the outcome of the infection as noted in the pathogenesis section. McCollum and Timoney (1999b) summarised the severity of clinical signs induced by different virus isolates. Thus, the VB-53 strain, derived from the original 1953 Bucyrus outbreak by serial passages in horses, produced severe signs of disease. Others like the KY-84, ARIZ-87, Nebr-89 and Ill-93 strains produced moderately severe signs, whereas KY-63, PA-76, KY-77 and the vaccine strain ARVAC produced sub-clinical infections. Similarly, host factors and virus strain appear to be important in determining the outcome of SHFV infection. Macaques are severely affected displaying a hemorrhagic syndrome which is often fatal, whereas patas monkeys experience mild and subclinical chronic infection or an acute infection depending on the strain they are infected with (Gravell *et al.*, 1986a).

Infection of pregnant mares can result in abortion, which has a serious economic impact for the horse breeding industry. It usually occurs 10-33 days post-

infection and can occur without the mare displaying any clinical signs (Timoney, 2002). Abortions have been reported in mares between 3 to 10 months of gestation. Mares do not appear to suffer fertility problems after EAV infection but stallions experience a reduction in the quality and quantity of the sperm during the acute phase of the disease but no fertility problems have been observed in persistent carrier stallions (Timoney, 2002). A proportion of stallions become chronically infected with EAV after the acute phase and can continue to excrete virus in the semen for months or years without displaying any signs of disease. The virus is excreted in the sperm rich fraction.

1.7. Diagnosis

Due to the asymptomatic nature of many natural infections and the fact that several of the clinical signs which may be presented are non-specific, it is always necessary to confirm suspected EAV cases by the laboratory.

1.7.1. Virus detection

Virus isolation from clinical specimens has been performed successfully using various cell lines such as LLC-MK₂, Vero, BHK-21 and primary equine dermal cells, but it appears that the most sensitive cell line and the most commonly used is RK-13 (Timoney, 2000). It appears that the passage number affects the sensitivity of this particular cell line with the technique being more successful when the cells are of low passage. Clinical specimens can include heparinised blood, serum, nasal swabs and conjunctival swabs collected during the acute phase of the infection, homogenates of various tissues, in particular lymph nodes, when biopsy samples are available and placental tissues and fetal fluids from

abortion cases. Also, virus isolation can be attempted from semen samples of persistently infected stallions. Confirmation of the presence of the virus in the clinical sample is made by observation of CPE in cells, which is characterised by rounding up and detachment from the vessel surface. Isolations have been successful with or without using a carboxymethylcellulose overlay (Timoney, 2000). Sometimes the CPE is not evident after the first passage and two or three blind passages are necessary before considering a sample negative. This is applicable in particular to semen samples. Identification of the isolates is made by a one-way virus neutralisation test, RT-PCR or by immuno-staining using monoclonal or rabbit polyclonal antibodies specific for EAV antigens, eg G_L and N (Balasuriya *et al.*, 1993; 1995a; Glaser *et al.*, 1995; Deregt *et al.*, 1994; Weiland *et al.*, 1999) as exemplified in a recent study (Balasuriya *et al.*, 2002b). Antibodies with these specificities have been used successfully to confirm the presence of viral antigen in tissues (MacLachlan *et al.*, 1996; Wada *et al.*, 1994; Lopez *et al.*, 1996). Isolation of EAV from clinical material is not always possible and is time consuming, in particular for semen samples. Failures to grow the virus from samples collected during the acute phase of the disease have also been reported (Monreal *et al.*, 1995). Detection of persistently infected virus shedding stallions can also be made indirectly by test-mating. The suspected stallion is mated to two seronegative mares on two consecutive days and detection of a rise in VNAb titre of the mares is taken as an indication of the shedding status of the stallion. These method, however is laborious and expensive and results are not available for at least 21 days.

Due to these inconveniences, molecular diagnostic methods have become increasingly popular in the last 10 years for the detection of EAV from clinical samples. The potential of these techniques for EAV diagnosis was demonstrated by Chirnside and Spaan (1990) who amplified in a single round RT-PCR reaction, single-stranded cDNA fragments from three conserved regions of the EAV genome. This technique had a sensitivity to detect 60 PFU / 100 µl from EAV positive semen samples. Since then, modifications of these procedures and new developments on PCR based diagnostic techniques have been reported. St Laurent (1994) compared the sensitivity of various primer pairs and obtaining best results with primers from the ORF1b region and reached a sensitivity of 2 and 20 TCID₅₀ of virus per 100µl of culture fluid and semen respectively. The introduction of nested amplification procedures using ORF7 specific primers (Belak *et al.*, 1994) improved the sensitivity of virus detection by RT-PCR and even traces (<0.007 PFU / µl) of EAV could be detected from semen and other clinical samples. The value of molecular diagnostic methods has been documented further (Gilbert *et al.*, 1997; Sekiguchi *et al.*, 1995; Ramina *et al.*, 1999). More recently the introduction of real-time PCR methods to molecular diagnosis of viral diseases, which are capable of monitoring the progress of the PCR reaction, has enabled the quantification of the viral load in the samples and facilitated high sample throughput minimising cross contamination (Leuteneger, 2001). In these methods, an oligonucleotide probe labelled with a fluorogenic dye and a quencher molecule, anneals to the target DNA in a position between the amplifier primers. As the reaction progresses, the 5'-3' nuclease activity of the thermostable polymerase cleaves the dye producing a fluorescent signal which is proportional to the amount of PCR product. A real-time TaqMan

diagnostic test for EAV has been described recently (Balasuriya *et al.*, 2002b) capable of detecting 200 copies of viral RNA from semen samples and tissue culture grown virus.

1.7.2. Serological diagnosis

Retrospective diagnosis of EAV infection is made by detection of virus specific antibodies in serum from convalescent horses. Early serological diagnostic methods were based on plaque reduction neutralisation assays (Burki, 1965; Hylseth, 1969; McCollum, 1970) and complement fixation tests (Fukunaga and McCollum, 1977; Fukunaga *et al.*, 1993). The former assays, although sensitive and specific, are laborious and not suitable for screening of large numbers of samples and therefore they are not routinely used today. Complement fixation tests using live virus antigen and formalin inactivated antigen have proved useful for diagnosis. These are less sensitive than virus neutralisation tests and it appears that CF antibodies disappear rapidly from serum after infection. These observations have been perceived as a drawback of the CF test for diagnosis of EAV, however this tests could be useful for detection of recent infections.

By far, the most widely used serological test used today and the one that is prescribed for international trade by the Office International des Epizooties (OIE), is the virus neutralisation test performed on microtitre plates as described by Senne *et al.* (1985). This test is best performed using RK-13 as indicator cells and guinea pig or rabbit complement to enhance test sensitivity. Virus neutralising antibodies (VNAb) are detectable in equine serum as early as day 7 post-infection and persist for months or years. Therefore it is necessary to take

acute and convalescent serum samples to diagnose recent EAV infections using this test. The test, although widely used and suitable for large sample screening and automation, can present problems in its performance and discrepant results have been obtained by different laboratories. This problem was recognised and addressed by the EAV OIE reference laboratory in Europe through an harmonisation programme on diagnostic procedures for EAV to identify the sources of variability of results produced by various laboratories (Edwards *et al.*, 1998). The two main factors identified affecting test variability were the use of rabbit or guinea pig complement and the virus strain. The CVL-Bucyrus strain, as held by the European EAV OIE reference centre (CVL, Addlestone, Surrey, UK), was found to increase the sensitivity of the test.

Due to the inconveniences of the VN test, alternative diagnostic procedures have been investigated. Enzyme-linked immunosorbent assays (ELISA) using antigen preparations derived from tissue culture grown virus have been developed (Lang and Mitchell, 1984; Cook *et al.*, 1989) and used in serosurveys, but in both cases false positive reactions occurred due to antibodies directed against tissue culture components present in sera of horses vaccinated with tissue culture derived preparations. Diagnostic ELISA procedures for EAV were further refined by the use of EAV N and G_L antigens expressed in *E coli* as recombinant glutathione-S-transferase (GST) proteins and used as the antibody capture component of the procedure (Chirnside *et al.*, 1995a; c). The GST-G_L (aa 55-98) based ELISA presented sensitivity and specificities of 99.6 % and 90.1 % respectively when compared to the VN test indicating that it is a useful diagnostic tool for primary screening of samples. Some of the field samples tested positive by ELISA but

negative by the VN test, but similarly sera collected from horses vaccinated with the killed vaccine showed a higher proportion of positives by ELISA than VN test, suggesting that the discrepant results could be due to higher sensitivity of the ELISA test. One of the inconveniences of the use of GST fusion proteins as antibody capture antigens was that some sera recognised this antigen non specifically. Thus, other G_L based antigens not comprising the GST molecule were evaluated as diagnostic antigens (Nugent *et al.*, 2000) in comparison to the GST-G_L protein. These studies demonstrated that a G_L derived synthetic peptide (aa 81-106) conjugated to ovalbumin presented less background reactivity than the other two antigens when used in an ELISA and that using a panel of 800 sera the G_L-OVA ELISA gave sensitivity and specificity values of 96.75 % and 95.6 % respectively. Similar tests based on G_L derived peptides have been used (Kondo *et al.*, 1998). Alternatively, baculovirus expressed N, M and G_L proteins have been demonstrated to be useful diagnostic tools either in immunoblotting (MacLachlan *et al.*, 1998; Kheyar *et al.*, 1997) or ELISA tests (Hedges *et al.*, 1998).

1.8. Immune responses to EAV infection

All the knowledge about the immune response to EAV, prior to studies of this thesis, was concerned with the development of virus specific antibodies which can be measured by VN test, CF test or ELISA. Experimental infections indicate that neutralising antibodies in serum appear within the first week after infection (Fukunaga and McCollum, 1977; Fukunaga *et al.*, 1981; McCollum, 1969, 1970, 1986; Paweska *et al.*, 1995) peaking on the second or third week and remain usually at high levels for months or years. These findings obtained from

experimental studies are confirmed by field data showing that horses in endemic areas maintain a high antibody titer over years, although this could be due to repeated infections as antibody titers correlate with the age of the animal (Burki *et al.*, 1992). Likewise, antibodies can be detected by ELISA soon after infection and are still detectable a long time after. Several data indicate that VNAb's play an important role in protection against and recovery from infection. Thus, the presence in serum of VNAb coincides with clinical recovery in experimentally infected animals (Fukunaga *et al.*, 1981); passively acquired maternal antibodies, which last between 2 to 8 months in foals (McCollum, 1976; Hullinger *et al.*, 1998) prevented or moderated EAV infection; and horses vaccinated with the formalin inactivated vaccine show various degrees of protection which correlated to the VNAb at the time of challenge (Fukunaga *et al.*, 1990, 1992). Infection with EAV results in a long-lasting, if not life-long, immunity. However, although recovered horses do not show signs of disease, they are rarely protected from re-infection, and sterile immunity is difficult to achieve in every circumstance with either inactivated or live vaccines (Doll *et al.*, 1968; Harry and McCollum, 1981; McCollum 1969a and 1969b, McCollum *et al.*, 1986, 1987; Fukunaga *et al.*, 1990; 1992; 1997). The mechanisms by which EAV specific antibody neutralises virus infectivity were addressed by three studies (Radwan and Burger, 1973a; b; Radwan and Crawford, 1974) showing this was mediated by physical interference of virus entry at high antibody concentrations and lysis of antibody coated virions by complement.

To date, very little is known about cell-mediated immune responses against EAV. In contrast, cellular immunity has been studied in other equine viral

infections like equine influenza, equine herpesvirus and notably equine infectious anemia contributing to gain further understanding of their pathogenesis and immunity. Thus, CTL precursors have been demonstrated in blood of equine influenza infected ponies after secondary stimulation *in vitro* in the presence of low concentrations of exogenous IL-2 (Hannant and Mumford 1989). Similarly, CTL assays have been developed to study EHV-1 immunity (Allen *et al.*, 1995) demonstrating that MHC-I restricted CD8⁺ T lymphocytes could be detected from the peripheral blood of convalescent horses and probably play an important role in controlling EHV-1 infections. This assay was modified later (O'Neill *et al.*, 1999) to determine the frequency of CTL precursors in peripheral blood demonstrating that high CTL precursor frequencies increase in ponies after infection with EHV-1. Equine infectious anemia virus infections have been the subject of extensive cellular immunology studies and methods to assess CTL responses and lymphoproliferation assays are well developed (McGuire *et al.*, 1994; 1997; 2000; Zhang *et al.*, 1998; Lonning *et al.* 1999a; 1999b; Hammond *et al.* 1997; 1998). The role of CTL's in control of virus infection was suggested after finding that activated CD8⁺ T lymphocytes could be detected in blood of EIAV infected horses and disappear from the circulation once the initial virus replication and plasma viraemia cease (McGuire *et al.*, 1994). However, CTL memory cells can still be detected from these animals, with Env or Gag/Pr proteins identified as the predominant CTL targets recognised. Fine mapping of viral protein targets for CD4⁺ and CD8⁺ CTL lymphocytes demonstrated that proteins p15 and p26 from the Gag viral protein contain peptides frequently recognised by EIAV infected horses opening the possibility of designing synthetic peptide vaccines against this disease, although it was also demonstrated

that it is unlikely that all horses recognise the same CTL peptide antigens due to differences in MHC-I haplotypes.

Similarly, immunological research of other arteriviruses has progressed more rapidly than that of EAV and, in particular for PRRSV and LDV, aspects of humoral, cellular and innate immunity have been addressed. Thus, pigs infected with PRRSV develop VNAb's after infection but the kinetics are somewhat different to those described in EAV. These VNAb's do not appear in serum until 4 weeks post-infection, although virus specific IgG are readily detectable by ELISA by day 7 post-infection (Murtaugh *et al.*, 2002). Cellular immune responses have been demonstrated in PRRSV infections in pigs and a role in final virus clearance can be attributed to these responses. Thus, Samson *et al.* (2000) observed an increase in CD4⁺ / CD8⁺ T lymphocytes in lungs of PRRSV infected pigs, PRRSV-specific T-lymphoproliferative responses have been detected in blood 4 weeks after infection (Lopez Fuertes *et al.*, 1999) and IFN- γ secreting T cells have been detected in lungs, lymph nodes and PBMC of PRRSV infected pigs (Lopez Fuertes *et al.*, 1999). However, persistent infections occur and pigs finally eliminating virus from oropharynx up to 150 days after infection have been observed. The mechanisms of immune evasion have not been elucidated for PRRSV but the findings of Suradhat *et al.* (2003) in peripheral blood collected from PRRSV infected pigs demonstrating an upregulation of IL-10 expression suggest that the virus might utilise the immunosuppressive effects of this cytokine to avoid an effective cellular immune response that would have otherwise cleared the infection faster. Consistent with this hypothesis is the observation that the frequency of IFN- γ secreting cells in PRRSV infected pigs

ranged from 50 to 100 / 10⁶ PBMC at 13 weeks post-infection or at 8 weeks post-vaccination whereas frequencies > 400 / 10⁶ PBMC have been observed just 2 weeks after administration of a pseudorabies vaccine (Murtaugh *et al.*, 2002).

Indications of a protective role for cytotoxic T lymphocytes in arterivirus infections have been obtained indirectly by experimental infection studies performed with SHFV in monkeys. Persistently infected patas monkeys can clear a subsequent infection with a different strain that is not neutralised by sera from these monkeys (Gravell *et al.*, 1986a). In contrast, LDV infection of mice induces a CTL response that has been reported to disappear by day 30 post-infection (Even *et al.*, 1995) or that persists up to day 250 post-infection (van den Broek *et al.*, 1997) which cannot prevent the establishment of a life-long persistent LDV infection. This virus seems to also evade the antiviral actions of innate immune defense mechanisms mediated by NK cells which become activated, showing increased expression of IFN- γ and cytolytic activity, but failed to alter the early and rapid LDV replication (Markine-Goriaynoff *et al.*, 2002). Another example of arterivirus interference of natural immunity is provided by the fact that IFN- α , which inhibits *in vitro* growth of PRRSV, is not produced during PRRSV *in vivo* infection (Albina *et al.*, 1998) and that PRRSV superinfection of coronavirus infected macrophages prevent these from producing IFN- α .

1.9. Current vaccines for Equine Viral Arteritis

1.9.1. Equine antiviral vaccines

Induction of immunity against viral diseases by traditional vaccination protocols is based in general in the inoculation of a virus which has been attenuated by

repeated passage in cell culture or laboratory animals, or by intramuscular injection of a virus inactivated by physical (usually high temperature) or chemical (formalin, β -propiolactone) methods and often formulated with an adjuvant.

As with other viral diseases of humans and other domestic animals, vaccination has often contributed to improve the health of horses worldwide. For example, inactivated vaccines were first developed to prevent equine influenza and have been used extensively for more than 30 years. These vaccines are formulated with oil or polymer adjuvants such as carbopol (Wood *et al.*, 1983; Mumford *et al.*, 1994b), or more recently with the adjuvant Quil A to form immunostimulating complexes (ISCOM's) (Mumford *et al.*, 1994) and provide different degrees of protection against infection and disease, which correlated with the pre-infection antibody levels measured by single radial hemolysis (Wood *et al.*, 1983; Mumford *et al.*, 1988). However, the antibody response is short-lived and frequent revaccinations are required to sustain an acceptable degree of immunity. Antigenic variation is another factor affecting the efficacy of vaccination procedures for equine influenza and it is important to update the antigenic content of the vaccines by incorporating the strains currently circulating in the field. Modern approaches for vaccination against equine influenza try to mimic the immune responses of natural infections, which confer long term protection which is not dependent on circulating antibody. Thus, cold-adapted live influenza vaccines have been studied and shown to have great potential for prophylaxis against this disease (Youngner *et al.*, 2001). Also, the growing understanding of the mucosal immune system (Hannant *et al.*, 2002) and the demonstration of a mucosal immune response following natural infection

(Nelson *et al.*, 1998) directed research towards mucosal vaccination. Thus, intranasal vaccination with inactivated equine influenza virus and the mucosal adjuvant CTB (the non-toxic B chain of cholera toxin) induced virus-specific IgA and neutralising antibody in the nasopharynx as well as serum IgG (Hannant *et al.*, 1999).

EHV-1 induced respiratory disease and abortion has been prevented by using live vaccines which were derived from the hamster adapted strain (Rac-H strain) which was subsequently passaged in equine cells (Mumford and Hannant, 1993). Live vaccines, used extensively in Europe and America, were useful in preventing abortion but reports of posterior paresis in horses vaccinated with attenuated EHV-1 vaccines (Liu *et al.*, 1977) indicated that virulence could be restored. Due to the difficulties in obtaining a safe attenuated vaccine vaccine research focused on the development of inactivated EHV-1 vaccines. Early studies with formalin inactivated EHV-1 vaccines formulated with complete Freund adjuvant demonstrated the potential of this strategy (Thompson *et al.*, 1978). However, this adjuvant is unsuitable for use in horses and killed vaccines formulated with other adjuvants did not prevent completely clinical signs or virus excretion. Nevertheless, inactivated vaccines dominate the market and there are reports that their use in a wide scale reduced the number of abortions due to EHV-1. These vaccines often include inactivated EHV-4 virus. Modern vaccination strategies for EHV-1 and EHV-4 infections include the use of sub-unit vaccines, often formulated as ISCOM's (Cook *et al.*, 1990; Hannant *et al.*, 1993), use of thymidine kinase deletion (TK⁻) mutants (Cornick *et al.*, 1990) and recombinant vaccinia virus encoding single EHV-1 proteins (Guo *et al.*, 1989;

1990), which has served to elucidate the protective role of individual virus components.

Prevention of African Horse Sickness has relied upon the use of live attenuated vaccines, by virus adaptation in mouse brain or in tissue culture. These vaccines are effective for protection against homologous strains but concerns have been raised about their safety. Thus inactivated vaccines have also been developed (van Dijk, 1999).

Other examples of conventional vaccines for horses are those developed for the prevention of arboviral encephalitides caused by members of the family *Flaviviridae* such as Japanese encephalitis virus or West Nile virus (Monath, 2001) or by members of the family *Togaviridae* such as Eastern equine encephalitis, Venezuelan equine encephalitis and Western equine encephalitis (Ostlund, 2000). Inactivated vaccines are generally preferred and these vaccines are prepared by growing the virus in tissue culture or mouse brain which is subsequently inactivated with formalin or monoethylamine.

1.9.2. Vaccines for EAV

Available EAV vaccines have been developed by classical methods. Currently there are two commercially available vaccines for the prevention of equine viral arteritis. The live attenuated vaccine was developed in the early sixties when techniques to cultivate EAV in tissue culture were established (McCollum, 1961). The virus was passaged serially in primary horse kidney cells (HK) and it was not found avirulent until passage 46. The virus was passaged further in HK

cells, primary rabbit kidney cells (RK) and equine dermal cells to optimise the safety and immunogenic properties of the virus for use as a vaccine. Thus, modified viruses with different passage histories have been used in different studies to evaluate these two properties. Most viruses studied were passaged 131 times in HK cells followed by 80 or 111 passages in RK cells. The last versions of the modified virus had 10, 16 or 25 extra passages in equine dermal cells. The commercial live vaccine Arvac is HK-131 / RK-111 / ED-24 passage virus.

It has been shown consistently over the years that inoculation of horses with tissue culture passaged viruses induce a transient leucopenia and mild febrile responses (Doll *et al.*, 1968; McCollum 1969; McCollum 1981; Harry and McCollum 1981; McCollum 1986; Fukunaga *et al.*, 1981; McKinnon *et al.*, 1986; Timoney *et al.*, 1988). Furthermore, virus has been detected in the blood of 12 out of 19 (Timoney *et al.*, 1988) and in 4 out of 9 (Fukunaga *et al.*, 1981) horses inoculated with the live vaccine, and from cervical lymph nodes of every animal up to day 8 post-vaccination (McCollum, 1981). However, these results contrast with those of McKinnon *et al.* (1986) who failed to detect virus from the buffy coat from a group of 16 vaccinated stallions. Isolations from nasal and rectal swabs have also been reported (Fukunaga *et al.*, 1981). The virus titres of the vaccination isolates were generally very low and considered insufficient to result in aerosol transmission. Indeed, earlier studies by Doll and colleagues (1968) showed that seronegative ponies in contact with vaccinated horses failed to develop an immune response and that five sequential passages in horses of the vaccine virus failed to restore pathogenicity. The vaccine virus is considered safe for use in stallions since these animals do not excrete virus in semen and no

alterations in fertility have been observed (Timoney *et al.*, 1988). In contrast, its use in the pregnant mare is not recommended since earlier attenuated versions inoculated into foetuses in utero induced abortion and virus could be isolated from various foetal tissues (Doll *et al.*, 1968). More recently, Moore *et al.* (2003) described a virus with an identical sequence to the modified Bucyrus vaccine strain that was derived from an aborted fetus whose dam was vaccinated a few days earlier with the live vaccine.

The immune responses following vaccination are characterised by appearance in serum of VNAb which are of low titre and can decline rapidly (Fukunaga *et al.*, 1981; McKinnon *et al.*, 1986; Timoney *et al.*, 1988) and therefore revaccination is recommended to sustain a protective degree of immunity. The anamnestic response after challenge is very good and clinical protection and reduction of duration of viraemia and virus nasal shedding against challenge has been demonstrated even after several months post-vaccination. However, sterile immunity is not always achieved even in efficacy studies where ponies were infected shortly after vaccination. Thus, horses challenged at 3, 6, 12, 18 and 24 months after vaccination with the HK-131 / RK-111 / ED-16 virus showed mild or no clinical signs, but challenge virus was recovered from the nasopharynx of almost all vaccinees and also on various occasions from the buffy coat (McCollum *et al.*, 1986). In another study, mares infected with semen from a persistently shedding stallion 19 days after vaccination with Arvac were clinically protected against equine arteritis but displayed febrile responses and virus was isolated from the nasopharynx and from the buffy coat for a few days (McCollum *et al.*, 1988). Similar results were obtained with horses challenged 8 weeks after being immunised with lyophilised preparations of HK-131 / RK-111

/ ED-16 virus (McCollum *et al.*, 1981). These apparent drawbacks have not prevented the use of Arvac successfully for the control of EAV in the states of Kentucky and New York (Timoney and McCollum 1988; Timoney, 1992). Prevention of PRRSV by use of a live modified vaccine has proved problematic since the vaccine virus could be excreted and transmitted to non-vaccinated animals giving rise to new genetic variants restoring virus pathogenicity (Oppriesnig *et al.*, 2002).

The discussed drawbacks of the EAV live vaccine, stimulated efforts to develop an inactivated vaccine which could be more suitable in non-endemic countries. Polyethylene glycol concentrated virus, produced in an equine dermal cell line and subsequently inactivated with 0.1 % formalin was demonstrated to be immunogenic in horses after two intramuscular inoculations in the absence of adjuvant (Fukunaga *et al.*, 1984). The animals receiving two doses 4 weeks apart develop VNAb titres as high as 1280 and 5120 one week after the second dose and were still seropositive (VNAb titres of 40 and 80) six months later. It was demonstrated in later studies that the duration of immunity, assessed by VNAb titre in serum, was improved by supplementary administration of the vaccine carried out 2 or more months after the basal dose regime (Fukunaga *et al.*, 1990). Clinical protection against challenge in these horses appeared to depend on the antibody titre at the time of challenge rather than on time after the last vaccination and it was concluded that the mean protective dose was 1: 43 and that 1: 80 was the 100% protective dose. This vaccine could be useful to prevent the carrier state since horses challenged two weeks after vaccination presenting VNAb titres > 1: 80 did not develop a viral infection of the reproductive tract

(Fukunaga *et al.*, 1992), and to prevent disease following venereal infection (Fukunaga *et al.*, 1997) and EAV abortion (Fukunaga *et al.*, 1996). A commercial inactivated vaccine (Artervac, Fort Dodge) is available in the UK although very limited data about efficacy and duration of immunity exists. The vaccine claims to stimulate VNABs after two doses but some field studies and experimental observations showed that detectable titres are not achieved after three or more inoculations (Castillo-Olivares *et al.* unpublished observations; Cardwell *et al.*, 2002).

1.10. Control of Equine Viral Arteritis

As with many other diseases of domestic animals control relies on combining serological surveillance, vaccination and sound animal management practices. For EAV, special emphasis is put on prevention of the carrier state, detection of persistent shedding stallions and prevention of EAV induced abortion. However, the policies to successfully achieve this goal vary between different countries, these differences motivated in great part by the differences in disease incidence and seroprevalence. Thus, the American Horse Council Protocol on EAV base its recommendations for the control of EAV on the extensive use of the live vaccine, permitting the breeding of mares to shedding stallions under special circumstances. The key aspects of this protocol are: a) vaccination of seronegative mares at least 21 days prior to breeding and if the stallion is shedding virus the mare should be isolated for at least 21 days; b) no vaccination of pregnant mares; c) revaccination annually of seropositive mares; d) testing all seropositive stallions for virus shedding; e) vaccination of seronegative stallions and annual revaccination but they should not be used for breeding for 30 days

after vaccination; e) seropositive shedding stallions can be used for breeding under the circumstances detailed above; f) seropositive non-shedding stallions with written certification of their seronegative status prior to vaccination need not be tested for virus shedding and should be revaccinated annually.

The live virus vaccine has not been used in Europe or Japan. Instead, the inactivated adjuvanted vaccine (Artervac, Fort Dodge) or the formalin inactivated preparation are available for the prevention of equine viral arteritis. The control measures implemented in the UK, an area of very low prevalence, contrast to those observed in the United States. In the UK, under the Equine Viral Arteritis Order 1995, EAV is a notifiable disease when a stallion is suspected of being infected or when a mare becomes infected after insemination (either by a stallion or by artificial insemination). Shedding stallions are not allowed to be used for breeding. The Horserace Betting Levy Board recommendations for the control of EAV are based on thorough serological screening of mares and stallions every year before the breeding season to restrict breeding practices to all seronegative animals and those seropositives demonstrated to be non-infectious. Seropositive stallions are tested by virus isolation from their semen or by test mating and only allowed for covering if negative. Seropositive mares need to show stable or declining VNAb titres with respect to previously tested samples in order to be regarded as non-infectious. The commercial inactivated vaccine can be used under an Animal Test Certificate on stallions. Therefore it is of paramount importance that the serological monitoring of every animal and stallions in particular is accurately recorded to determine whether a positive VN result is due to vaccination or infection.

None of the vaccines described above permit serological discrimination of infected from vaccinated animals. Such discrimination would allow the use of vaccination without compromising serological surveillance in non-endemic areas, such as the UK, that are at risk of infection due to constant movement of animals or would be useful in the implementation of eradication programmes should the disease becomes endemic. Disease eradication programmes based on the use of marker vaccines for Aujeszky's disease of pigs or bovine infectious rhinotracheitis have been applied in Germany and the Netherlands (van Oirschot, 2001).

1.11. Alternative approaches to conventional vaccines for the prevention of viral diseases.

The advent of molecular biology and recombinant DNA technologies permitted the advance of medicine, immunology, virology and opened new avenues for the generation of safer and more efficacious vaccines and facilitated the design of marker vaccines. These lack an antigen that is present in the pathogen causing the disease they intend to prevent, or contain an additional antigen absent from the pathogen in its natural form. The result is that naturally infected animals develop an antibody response of different specificity to that resulting from vaccination (for reviews see Babiuk, 1999; van Oirschot, 2001; Collet, 1989).

1.11.1. Sub-unit vaccines

These vaccines are based on a single or a discrete number of proteins from the pathogen identified as relevant for immune protection. These can be produced by conventional means as is the case for the production of tetanus toxoid by

growing the bacteria in fermenters and subsequent purification and inactivation of the toxin. But also these vaccines can be produced by chemical synthesis of polypeptides or by using recombinant DNA technology by cloning the desired gene into the appropriate vector for subsequent production of the immunogen in an expression system. Various expression systems have been used for the production of sub-unit vaccines, from bacteria (*E coli*, *Bacillus spp*), yeast (*Saccharomyces spp*, *Piscchia spp*) or baculovirus (*Autographa californica* nuclear polyhedrosis virus) to mammalian cells. Bacterial systems, in particular *E coli*, have been used extensively since they are well characterised and large quantities of the protein can be produced and purified. However, the general view is that they may not be the best choice for the production of viral proteins whose tridimensional conformation is essential for maintainance of their antigenic properties, since bacteria do not modify the proteins post-translationally. Therefore an eukaryotic cell system might be preferred. Expression in yeast has the advantage of cost since there is extensive experience in growing yeasts in an industrial setting and the expression levels are very high compared to mammalian expression systems. This system has not been widely used for the production of veterinary vaccines but the first sub-unit vaccine against Hepatitis B was and is still produced in yeast (Valenzuela *et al.*, 1982). The baculovirus expression system offers the advantage that the desired genes can be cloned into the virus genome under the control of very a strong promoter such as that of the polyhedrin protein, a non-essential protein which is expressed abundantly in baculovirus infected cells. However, production is difficult to scale up. The system has been used extensively as a vaccinology research tool and in some cases good prospects exist for the commercial use of baculovirus expressed

immunogens as is the case for African Horse Sickness (Martinez-Torrecuadrada *et al.*, 1994; 1996; Roy *et al.* 1996; du Plessis *et al.*, 1998; Scanlen *et al.*, 2002).

Mammalian expression systems are often the best option for producing correctly folded proteins. In these approaches, the desired gene, cloned downstream of an appropriate promoter, is introduced into mammalian cells via a viral vector (e.g. vaccinia, herpesvirus, adenovirus, Sindbis, polio) which then is translated and the protein produced, purified and used as an immunogen. These systems are often difficult and costly to scale up in an industrial setting because the cells are lysed by the virus or need to be lysed to release the protein from the cell. This means the recombinant immunogen need to be purified away from cell proteins and viruses. Furthermore, some proteins have toxic effects to the cells. For these reasons, alternative systems have been developed for the production of sub-unit vaccines in mammalian cells. These are based on the generation of cell lines stably transfected with genes controlled by inducible promoters that encode proteins that can be secreted into the medium therefore simplifying purification steps. Thus, Kowalski *et al.* (1993) cloned the gB gene of bovine herpesvirus-1 devoid of the transmembrane region under the control of a strong promoter such as bovine HSP70. The cells are scaled up first and then the temperature is switched to 43°C to drive the expression of gB which is secreted in the medium facilitating its purification.

Synthetic peptide vaccines have also been used as sub-unit vaccines. Since these molecules are very small they are normally coupled to large carrier molecules such as keyhole limpet hemocyanin (KLH) or ovalbumin in order to provide

sufficient T helper epitopes to stimulate a strong immune response across a wide range of MHC antigens in a population. Promising efficacy data were obtained with experimental synthetic peptide vaccines against various animal virus diseases including foot-and mouth disease (Bittle *et al.*, 1992) although these did not meet the requirements for the existing classical vaccine. The main problems are associated with antigenic variation and mimicking conformational epitopes of antigens. More success has been obtained with vaccination against canine parvovirus infection of dogs using a synthetic peptide based on a linear and conserved B cell epitope of the virus coat (Langeveld *et al.*, 1994).

Sub-unit vaccines therefore have the advantages of safety, stability, being chemically defined and importantly having 'marker' capacity. The main disadvantages are their low immunogenicity and rapid degradation *in vivo*. It is often necessary to incorporate adjuvants in their formulation as with inactivated vaccines.

Adjuvants are chemical compounds which combined with the immunogen of choice enhance the immune response after administration. The modes of action of adjuvants can be classified, according to Cox and Coulter (1997), into the following categories: a) Immunomodulation: refers to the capacity of the adjuvant to direct the immune response towards either a Th1 or Th2 type response; b) Presentation: refers to the way cells of the immune system recognise the antigen; c) Induction of CTL; d) Targetting: the adjuvant affects the way the antigen is taken up by antigen presenting cells; e) Depot generation: slows down the *in vivo* degradation of the antigen. A vast number of different compounds have been used as adjuvants although only very few have been licensed for use in

commercial human and veterinary vaccines. Thus, aluminium hydroxide, has been for a long time the only licensed product for use in human vaccines, is a safe, inexpensive gel-like particulate adjuvant which possesses good targeting properties inducing a strong Th2 type response with elevated antibody production, moderate depot effect and stimulating very poor cellular immunity. Freund's complete and incomplete adjuvants, used extensively in monoclonal antibody production, are water-in-oil emulsions. These are microdroplets of water stabilised in a continuous oil layer (mineral oil, squalene, squalane or metabolizable oil). The targeting and presentation are poor whereas the CTL induction and depot effects can be strong. Oil-in-water emulsions are oil microdroplets stabilised by surfactants in a water phase which can contain immunomodulators such as muramyl-dipeptide or block copolymers. They are suited for amphipatic molecules and have good presentation properties. Immunostimulating complexes (ISCOM's) are cage-like structures resulting from the interaction of saponins with cholesterol and phospholipids and induce strong Th1 and Th2 responses with good CTL and excellent presentation. They have been used in veterinary vaccines and their use in human vaccines is currently being considered. Other type of adjuvants include liposomes, nanoparticles, muramyl dipeptide and derivatives, non-ionic block copolymers, saponins, cytokines, and CpG motifs.

1.11.2. Recombinant live virus vaccines.

Advances in recombinant DNA technology allow with relative ease the manipulation of viral genomes for the generation of vaccines. The viral genomes

can be modified to produce an attenuated virus (by deletion of virulence determinants) to immunise against the disease caused by the original virus or to deliver heterologous genes encoding the protective immunogens of other viruses. Thus, genes encoding the thymidine kinase (TK) or the glycoproteins gE, gG or gC of Aujeszky's disease and infectious bovine rhinotracheitis viruses were deleted or rendered inactive to generate attenuated virus vaccines (Kit *et al.*, 1985; Mettenleiter *et al.*, 1994). A step further in the development of this type of vaccines is the deletion of an essential gene rendering the virus unable to replicate in the host and therefore increasing vaccine safety. For the production of such deletion mutants it is necessary to supply the essential gene by a helper virus or by infecting a cell line stably transfected with the gene in question. This type of defective single cycle virus has been described for herpesvirus infections (Farrell *et al.*, 1994). With the development of reverse genetics, the manipulations described above have been possible for positive sense RNA viruses too, and thus full length cDNA clones of flaviviruses (Monath *et al.*, 2001), coronaviruses (Ortego *et al.*, 2002), alphaviruses (Pushko *et al.*, 1997), pestiviruses (Moorman *et al.*, 1996; van Gennip *et al.*, 2002) and arteriviruses (de Vries *et al.*, 2000; 2001; van Dinten *et al.*, 1997; Verheije *et al.*, 2001) have been created enabling the generation of vaccines for the diseases the parental viruses produce or against other diseases.

Indeed, attenuated viruses obtained either naturally, by conventional means or by recombinant DNA technology can be used as gene delivery vectors. The most extensively used virus vector is vaccinia virus. There are vast numbers of immunology, virology and vaccinology studies describing expression of

heterologous genes using the vaccinia virus (for a review see Paoletti, 1996) and only a few examples are cited here. Thus, the glycoprotein G of rabies virus was inserted into the thymidine kinase site of the Copenhagen vaccinia virus strain to generate a rabies vaccine which has been used successfully in the field for the prevention of wild-life rabies (Pastoret and Brochier, 1996). Other poxviruses have been used as vaccine vectors such as canarypox for the prevention of feline leukemia (Tartaglia *et al.*, 1993; Poulet *et al.*, 2003), equine influenza or EHV-1 infections (Minke *et al.*, 2000) or fowlpox virus to prevent rabies in dogs (Taylor *et al.*, 1995).

Examples of other attenuated viruses as vectors are bovine herpesvirus 1 encoding capsid epitopes of foot and mouth disease, human adenovirus 5 encoding the spike protein of porcine transmissible gastroenteritis and yellow fever 17D strain carrying the prM or E genes of Japanese encephalitis virus (Monath *et al.*, 2001). All these recombinant virus vaccines have most, if not all, the advantages of a live virus vaccine, providing cellular as well as humoral immunity and long duration of immunity. Additionally, they are safer and have the capacity to discriminate between vaccinated and infected animals.

Recombinant virus vaccines for EAV have also been devised. Thus, use of alphavirus replicon particles based on venezuelan equine encephalitis virus has been documented for the *in vivo* expression of G_L and / or M of EAV (Balasuriya *et al.*, 2000; 2002a) in mice and horses resulting in the stimulation of VNAb's when both proteins were expressed together but not when either protein was expressed alone. The immune responses generated in GL / M vaccinated horses conferred clinical protection, reduced virus excretion and prevented viraemia

against intranasal challenge infection. These levels of protection were slightly lower when the vaccinates were challenged by intra uterine virus inoculation.

The generation of a cDNA infectious clone of EAV will allow the deletion or insertion of genes to modify the replication properties, tissue tropism and/or antigenic specificity of the antibody response of EAV infection to generate improved EAV vaccines. Furthermore, a cDNA clone derived virus, has been found to be infectious, avirulent and genetically stable after inoculation in horses (Balasuriya *et al.*, 1999b) demonstrating the vaccine potential of this approach.

1.11.3. DNA vaccines

DNA vaccines are composed of bacterial plasmid DNA encoding the desired genes under the control of appropriate mammalian expression regulatory sequences. The DNA vaccines are administered to the animal by the intradermal or intramuscular route and these are taken up by various cells resulting in the expression *in vivo* of the immunogen which then can be presented to T lymphocytes via MHC-I and MHC-II molecules, and be recognised by B cells in the surface of the cell membrane or in soluble form if the protein is secreted. This approach has been tried in many veterinary species including cattle, pigs, cats, dogs and horses. The main disadvantage of this approach is the high dose of DNA required to achieve sufficient expression levels in the host. Often, potentiation of the immune response has been tried by incorporating cytokine genes, CpG motifs or classical adjuvants. Another issue is safety with regards to integration of the foreign DNA into the host genome.

DNA vaccination has been explored for EAV. Thus, immunisation protocols involving five inoculations in mice of 100 µg of plasmid DNA encoding ORF7, ORF5 or ORF5 ectodomain of EAV or a combination of plasmids encoding ORF2, ORF5, ORF5 ectodomain, ORF7 and equine IL-2 administered four times to horses were reported to stimulate neutralising antibodies (Tobiasch *et al.*, 2001; Giese *et al.*, 2002).

1.12. Aims of the thesis

The main objective of this research work was to explore the potential of two vaccination strategies to generate a marker vaccine for EAV as well as to develop an assay to measure cytotoxic T lymphocyte responses, which will assist in future determination of the contribution of CTL's to protective immunity and the efficacy of EAV vaccines.

Based on the findings by Chirnside *et al.* (1995a; 1995c) identifying a major immunodominant region on the EAV G_L protein containing sequences that stimulate a VNAb response in ponies after intramuscular inoculation, I have explored further the potential of the G_L sub-unit vaccination approach. Modifications of the vaccination protocol, used by Chirnside *et al.* (1995a), in particular changes in the amino acid composition of the immunising protein, were introduced in an attempt to increase the duration and strength of the antibody responses. The protective efficacy of these responses against a virulent challenge was evaluated.

An alternative 'marker' vaccination strategy based on a genetically modified mutant EAV virus was evaluated. The *in vivo* properties of an EAV G_L deletion mutant, the immune responses stimulated and their protective efficacy against a virulent challenge were studied.

Although VNAb titres in serum appear to be a correlate and an effector mechanism of immunity for EAV, nothing is currently known about cellular immune responses and the role they play in protection and virus clearance. Development of methods to measure CTL responses is described.

Attempts to identify viral proteins targetted by CTL's will assist the development of future vaccines and would help to elucidate whether CTL responses alone are sufficient to provide protection against infection. A gene delivery system based on a baculovirus expression vector was developed in order to express EAV genes (using N as a model) in equine primary dermal cells and to test whether this system could be used to identify CTL targets.

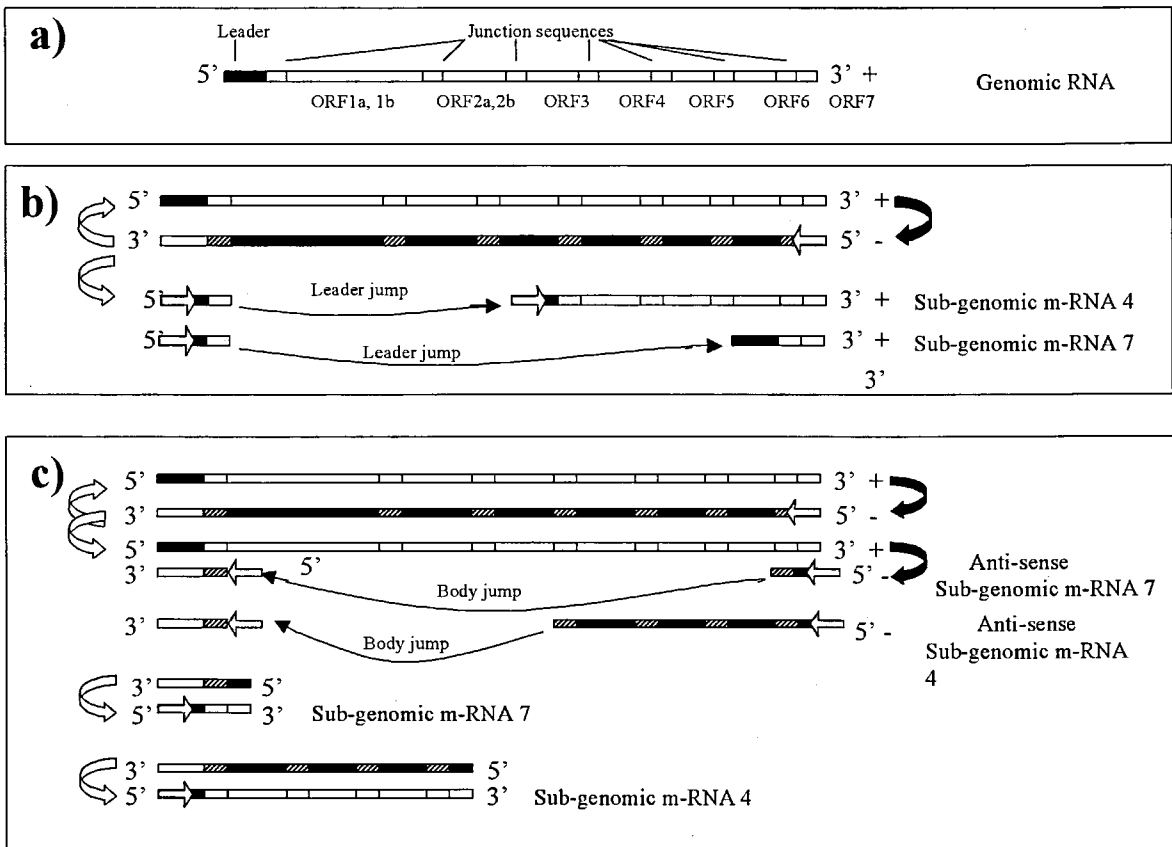


Figure 1.1. Schematic representation of two models of discontinuous EAV RNA transcription. Synthesis of m-RNA 4 and m-RNA 7 are illustrated. A) Representation of the genomic RNA. B) In this model the positive sense genomic RNA is transcribed to generate a full length negative strand which serves as a template for the generation of more genome length positive strands and the sub-genomic m-RNA's from which the 5'-most ORF is translated. The leader sequence, once transcribed, 'jumps' to any of the negative junction sequences of the template via base-pairing of the positive and negative sense junction sequences to prime the transcription of the sub-genomic RNA. C) The positive genomic RNA strands transcribed from the anti-sense genomic RNA are the template for anti-sense subgenomic RNA synthesis. In this case, transcription of the negative subgenomic RNA's is interrupted at the junction sequences and the body of the RNA jumps to the leader sequence of the positive genomic strand to complete the transcription generating the anti-sense leader sequence. The anti-sense subgenomic RNA's serve as templates for the synthesis of the sub-genomic m-RNA.

Chapter 2. Immunogenicity of EAVG_L ectodomain based polypeptides.

2.1. Introduction

The potential of EAV G_L ectodomain derived antigens to be used as a sub-unit vaccine for EAV has been demonstrated in previous studies in which serum VNAb were stimulated in ponies after intramuscular injection of Carbomer-PD adjuvanted vaccines (Chirnside *et al.*, 1995a). These comprised a synthetic peptide (aa 75-97) conjugated to keyhole limpet hemocyanin (SP25-KLH) and a glutathione-S-transferase fusion recombinant protein FP5RsaI (aa 55-98). However, peak VNAb titres varied between individuals and disappeared from serum relatively rapidly. In an attempt to increase duration and levels of serum VNAb antibodies after vaccination, various adjuvants, different doses and different EAVG_L based immunogens were tested. In addition to FP5RsaI and SP25-KLH, the following antigens were used: Pep1-KLH (aa 81-106), which included a neutralising antigenic site absent from previously tested polypeptides (aa 99-106), Pep2-KLH (aa 81-98) and a bacterially expressed polyhistidine tagged protein, 6hisG_Lecto (aa 18-122), which includes all regions identified as targets for neutralising antibodies (Balasuriya *et al.*, 1997; Chirnside *et al.*, 1995a).

2.2. Materials and Methods

2.2.1. Synthetic Peptides and Recombinant Proteins

All synthetic peptides and recombinant proteins used as immunogens in this study derive from the G_L amino acid sequence of the Bucyrus-Utrecht strain (den

Boon *et al.*, 1991). The GST fusion protein FP5RsaI (aa 55-98) and the keyhole limpet hemocyanin (KLH) conjugated synthetic peptide SP25 (aa 75-97) have been described previously (Chirnside *et al.*, 1995a). In addition, new immunogens were prepared. Peptide-1 (aa 81-106) and Peptide-2 (aa 81-98), obtained from Chiron Mimotopes (Victoria, Australia), were synthesized including an extra cysteine residue at the carboxy-terminus to allow coupling to KLH via the maleimide cross-linking moiety of sulfo-SMCC (*N*-succinimidyl 4-(*N*-maleidomethyl)cyclohexane-1-carboxylate). The Inject Activated Immunogen Conjugation Kit (Pierce, Rockford, IL, USA) was used for this purpose. In addition to the above peptides an N-terminally polyhistidine tagged recombinant protein covering the entire G_L ectodomain (aa 18-122), denoted 6hisG_Lecto, was also used. A batch of this protein was kindly supplied by Dr A.A.F. de Vries at the University of Utrecht. Cloning, expression and antigenic analysis of the 6-hisG_Lecto protein are described elsewhere (Nugent *et al* 2000). Briefly, the EAVORF5 gene fragment encoding the predicted N-terminal ectodomain of G_L (i.e. without the predicted signal sequence) was cloned into the pQE9 (Qiagen) expression vector, thereby fusing an N-terminal 6-His motif to the G_L ectodomain. Bacteria (*E. coli* strain M15) transformed with both pREP-4 and pQE9/G_Lecto were cultured in Terrific Broth medium containing 100 µg /ml ampicillin and 50 µg/ml kanamycin. Protein expression was induced by addition of IPTG to a final concentration of 2 mM after 1 hour incubation at 37°C. Following 5 hours induction, bacteria were pelleted, subjected to lysozyme digestion in the presence of protease inhibitors, treated with DNase and RNase and insoluble protein was pelleted by centrifugation (6000g, 15 min, 4 °C). The pellet was washed in distilled water and resuspended in 6M guanidine

hydrochloride, 20mM sodium phosphate, pH 7.8, 500 mM NaCl. The solubilized protein was then applied to a Ni^{2+} charged affinity column (ProBondTM column, Invitrogen) and subsequently eluted with 8M urea, 250 mM imidazole, 0.1 NaH_2PO_4 , 0.01 M Tris pH 6.0. The protein solution was stored in the dark at 4 °C until used.

All the vaccines were prepared in 2 ml doses and administered by the intramuscular route. The antigen stock solution was diluted in 1 ml of phosphate buffered saline (PBS) to the required concentration. Then, 1 ml of proprietary co-polymer adjuvant (Solvay-Duphar / Fort Dodge, Holland) was added. The doses of the GST-fusion protein FP5RsaI vaccine were calculated taking into account the molar ratio of the G_L antigen to the whole protein (5/32). The dose of the G_L antigen on the KLH-conjugated peptide vaccines were calculated considering that the 1: 1 KLH : peptide (mass) reaction occurred with an efficiency of 100%.

2.2.2. Analysis of G_L recombinant proteins by polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

The purified 6his G_L ecto and FP5RsaI proteins were diluted in 2x sample buffer (250 mM Tris-HCl, 2% SDS, 10% glycerol, 20 mM DTT, 0.01% bromophenol blue) and heated at 95°C for 4 minutes. The pre-stained SDS-PAGE Standards Broad Range (BioRad) was used as the molecular weight marker (MWM) according to manufacturer's instructions. The protein samples were run for 30-45 min at 160 Volt on 12.5% SDS-PAGE mini-gels immersed in Running Buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3) and stained with Coomassie blue solution (50% methanol, 10% acetic acid, 0.25%

Coomasie blue R-250). Gels were finally destained in destaining solution (40% methanol, 10% acetic acid).

Proteins on acrylamide gels were electroblotted onto nitrocellulose membranes (Hybond) previously wet on transfer buffer (25 mM Tris-HCl, 19.2 mM glycine, 20% methanol) applying a current of 7 Volt for 15 min. The membranes containing the electroblotted proteins were blocked overnight in 2% NFM PBST [PBS, 2% non-fat milk (BioRad), 0.05% Tween 20 (BDH)]. After washing in PBST, the membranes were incubated with anti-G_L specific antibodies α -SP25 (Chirnside *et al.* 1995a) or α -6hisG_Lecto, obtained by repeated intramuscular immunisation of a pony with this protein. These primary antibodies were diluted 1/1000 in 2% NFM PBST and incubated with the membranes for 1 hour at 37°C. After another wash in PBST, horseradish peroxidase (HRPO) conjugated anti-horse IgG (Pierce) or anti-rabbit IgG (DAKO) antibodies diluted 1/500 or 1/1000 were added to the membranes and incubated for another hour at 37°C. A final wash in PBST was performed and the membranes were incubated for 2 min with the HRPO chromogenic substrate TMB. The reaction was stopped with 0.18M H₂SO₄.

2.2.3. Measurement of Antibody Responses

Serum samples were collected regularly from the ponies and VNAb titres were determined according to Senne *et al.* (1985). Briefly, serial two-fold dilutions of heat treated serum (56°C, 30 min.) were carried out in microtitre plates (25 μ l/well) from a starting dilution of 1/2. The diluent was MEM [minimum essential medium Eagle with Earle's salts (Sigma, M2279) supplemented with

100 IU of penicillin per ml, 10 µg of streptomycin per ml, non-essential amino acids, L-glutamine to a final concentration of 2 mM and 200 mM HEPES] supplemented with heat inactivated foetal bovine serum to a final concentration of 5 %. Then 25 µl of a virus suspension (100 TCID₅₀) in medium containing 10% guinea pig complement was added to each well. The virus used (CVL Bucyrus) is a derivative of the Bucyrus strain obtained from the OIE Reference Laboratory, Weybridge, UK. This virus, a standard reagent for the VN test, has been evaluated in trials and found to give high sensitivity (Edwards *et al.*, 1998). Three control equine sera and a titration of the virus dose were included in each test. After 1 hour incubation (37 °C, 5% CO₂), rabbit kidney (RK-13) cells (100 µl/well, 1.2 x 10⁵ cells/ml) were added, the plates sealed and the results read after a 48 hour incubation period. Wells showing more than 50 % cytopathic effect were recorded as positive. Titres were calculated using the Karber formula (Karber, 1931) and expressed as log₁₀ VN₅₀.

Anti-G_L specific antibody responses were measured using the Peptide-1 based diagnostic ELISA test described by Nugent *et al.* (2000).

2.2.4. Experimental infections

Virus LP3A, provided by Mr David Westcott at the Veterinary Laboratories Agency, Surrey, England, is a large plaque variant derived from a pleural fluid isolate collected from a horse which died after being infected with the 14th horse passage of the original Bucyrus strain (McCollum *et al.*, 1971). Plaque purification and growth of virus stocks were carried out in primary equine embryonic lung (EEL) cells. All challenges were performed in a controlled environment building. Two vaccinates and an unvaccinated control pony were

used in each challenge. The ponies entered the building 1 week before the challenge to acclimatise to the new environment and remained in contact thereafter for 21 days. The ponies were examined twice a day every day while within the building. Infection with EAV was by instillation into the ponies' nasopharynx of 1.0 ml virus suspension containing 10^6 TCID₅₀ using a flexible tube with spray attachment. Symptoms and rectal temperatures were recorded from the day of challenge for a period of 14 days. Nasopharyngeal swabs collected over the 14 day period were stored into standard virus transport medium and frozen at -70 °C. Heparinised blood samples were processed immediately for virus detection.

2.2.5. *Virus isolation*

Swabs collected from experimentally infected ponies were thawed, squeezed and the resulting extract used for virus isolation. White blood cells from 8 ml of plasma collected from unclotted blood samples were pelleted by low speed centrifugation and resuspended in 2 ml of MEM. Procedures to isolate EAV were performed essentially as described previously (Paweska *et al.*, 1996). Volumes of 0.5 ml of nasal swab extract or white blood cell suspension were inoculated into 25 cm² flasks of confluent monolayers of RK-13 cells. The flasks were incubated for 1 hour at 37 °C, 5 % CO₂ and overlaid with 5 ml of MEM supplemented with 2% bovine serum (2 % MEM). The following day the medium was discarded, fresh medium added and the flasks incubated for 5-6 days. Confirmation of isolated virus was made by indirect immunofluorescence using a mixture of two monoclonal antibodies to the nucleocapsid protein kindly supplied by Dr Cordioli (Istituto Zooprofilattico Sperimentale, Brescia, Italy).

Samples were recorded as negative after failure to isolate EAV on a second passage. Nasal swab extracts were titrated as follows. Serial dilutions ($0.5 \log_{10}$) were made in quadruplicate in 96 well trays using a volume of 150 μ l. Then, 100 μ l of 2 % MEM containing 10^4 RK-13 cells were added to each well and incubated at 37 °C, 5 % CO₂ for 4 days. Wells presenting cytopathic effect were recorded as positive and virus concentration calculated according to Karber and expressed as TCID₅₀/ ml of swab extract.

2.2.6. Statistical analysis

Six variables have been used for the statistical analysis of the data: a) duration of pyrexia (rectal temperature >38.5 °C), b) maximum temperature, c) duration of viraemia (number of days virus was isolated from blood samples) d) duration of nasal excretion of virus, e) maximum virus excretion in one day and f) total virus excretion, calculated as the arithmetic sum of virus excretion values over the first ten days post-infection. The study was a standard randomized block experimental design (with challenge groups as blocks), with the vaccine treatment applied to two ponies and the control treatment to one pony in each group. For this experimental design the appropriate analysis of variance (ANOVA) model is: $X_{ij} = \mu + G_i + T_j + e_{ij}$, where X_{ij} = the observed variable value for the pony in i-th Group receiving j-th treatment, μ = overall mean value, G_i = i-th Group effect, T_j = j-th Treatment effect, and e_{ij} = random error term assumed to have normal distribution with mean = zero and constant variance. For each variable studied, the mean and standard deviation were calculated as well as the ANOVA to test the null hypothesis of no difference in the effects of the two experimental treatments (vaccination versus no vaccination), on the variable

concerned. The test for the null hypothesis $H_0: T_1 = T_2$ where T_1 = control and T_2 = vaccine, used the standard variance-ratio F-test with 1 and 11 degrees of freedom. Details of the 'Randomized Blocks' experimental design and analysis may be found in the following textbooks (Cochran & Cox, 1957; Scheffe, 1959; Steel & Torrie, 1980). The effect of pre-challenge VNAb titres upon subsequent virus shedding by the ponies was assessed using both correlation and regression relationship between pre-challenge titre values and total virus excretion.

2.3. Results.

2.3.1. Selection of 6hisG_Lecto as a potential sub-unit vaccine candidate

2.3.1.1. Experiment I

Four experimental vaccine preparations containing either Pep-1-KLH or FP5RsaI, adjuvanted with either Carbopol or Amplimune-Alhydrogel were tested in ponies. Two doses of 160 µg of each EAV G_L antigen, equivalent to 1 mg of FP5RsaI and 320 µg of Peptide-1-KLH, were administered four weeks apart by intramuscular injection to four ponies and antibody responses measured by the Peptide-1 ELISA (Nugent *et al.*, 2000) and the VN test.

All ponies responded to the first vaccine dose, developing antibodies to the Peptide-1 antigen (Figure 2.1). The serum antibody levels increased after the second vaccination, remaining at high levels up to week 8 and then declined progressively. There were little differences in the peak O. D. values of the ELISA antibody response between pony groups or between individuals. In contrast, differences in VNAb were more pronounced and only the Fp5RsaI / Carbopol preparation induced measurable titres, which peaked one week after the second vaccination and then declined rapidly to undetectable levels by week

10. These results are consistent with Chirnside's studies with the Fp5RsaI / Carbopol vaccine, despite the different vaccination regimes (v2 was given on day 28 instead of day 56) and doses (120 µg instead of 160 µg of EAV antigen). Interestingly, the FP5RsaI recombinant protein did not induce VN antibodies when it was used in combination with Amplimmune / Alhydrogel. Further vaccination experiments were performed with Carbopol adjuvant only. In contrast to the results obtained by Chirnside *et al.*, (1995a) showing a VNAb response following vaccination with SP25-KLH, in the present experiment ponies receiving the Peptide-1-KLH vaccine did not mount a VNAb response. The dose (160 µg), amino acid composition of the Peptide-1 (G_L aa 81-106) and method to conjugate the peptide to the KLH carrier molecule (malic anhydride linkage) were different to those used by Chirnside (60 µg, G_L aa 75-97, glutaraldehyde conjugation) and could have contributed to these results.

We tested further the potential of synthetic peptide vaccination for EAV by comparing the immunogenicity of the original SP25-KLH antigen preparation used by Chirnside with Peptide-1-KLH and Peptide-2-KLH.

2.3.1.2. Experiment II

Three groups of two ponies were vaccinated by the intramuscular route with Peptide 1-KLH, Peptide 2 -KLH or SP25-KLH on weeks 0, 4 and 11 using 62 µg of G_L antigen (124 µg of peptide-KLH conjugate), which was formulated with Carbopol. Antibody responses were analysed by the VN test and the Peptide-1 ELISA (Figure 2.2). All ponies developed a rapid IgG antibody response to Peptide-1 antigen, which remained very high throughout the study period for the Peptide-1-KLH and Peptide-2-KLH vaccinated groups. The intensity of the

antibody response of SP25-KLH vaccinates was weaker than in the other groups, especially after the first two immunisations. Sera began to show low levels of virus neutralising activity after the second dose but significant titres (>0.6) were not reached after the last boost on week 11. The Peptide-2-KLH vaccine group presented the highest titres (1 and 0.9). Neither the use of a shorter and less hydrophobic peptide (Peptide-2), different dosage nor administering the peptide vaccines three times produced higher peak serum VNAb titres than those obtained with the FP5RsaI / Carbomer-PD vaccine. As in the previous experiment, all three synthetic peptides elicited low titre VNAb's despite anti-Peptide-1 antibodies being easily detected, suggesting that conformation of the G_L protein plays an important role in preserving neutralising epitopes.

To investigate the influence of immunogen tertiary structure in virus neutralisation and the antigenic resemblance of vaccine neutralising epitopes to native G_L epitopes, anti-Peptide-2 specific antibodies were depleted from a serum sample collected from a naturally infected horse. The serum samples before and after antibody depletion were analysed for their reactivity to the Peptide-2 antigen by ELISA and for their virus neutralisation capacity in the VN test. For this, 10 µg of biotinylated Peptide-2 were incubated with streptavidin coated paramagnetic beads, used in excess (1.5 mg) to maximise Peptide-2 binding (binding capacity of beads is 10 µg peptide/mg of beads). Then 1/4 and 1/100 dilutions of the serum were incubated with the beads.

The depletion assay results (Figure 2.3) show that Peptide-2 specific antibodies were effectively removed from the samples since the ELISA absorbance readings for all serum dilutions from the depleted samples (both dilutions) were very low

in comparison to the undepleted ones. However, the antibody depletion procedure did not produce a decrease in VNAb titres, supporting the hypothesis that Peptide-2 does not mimic neutralising epitopes or that virus neutralisation depends on antibodies binding to other epitopes outside the Peptide-2 amino acid sequence.

The above results, the known conformational nature of certain virus neutralising epitopes in EAV G_L (Deregt *et al.*, 1994; Balasuriya *et al.*, 1997) and the presence of some of these epitopes in regions of G_L not included in either Peptide-1, Peptide-2 or SP25 led us to investigate the immunogenicity of a polyhistidine tagged recombinant protein, 6hisG_Lecto, expressed in *E coli* which comprised the whole ectodomain of G_L.

2.3.1.3. Experiment III

The antigenicity of the 6hisG_Lecto protein has been demonstrated previously in solid phase format immunoassays (Nugent *et al.*, 2000). A new batch of this protein produced specifically for immunisation experiments was kindly supplied by Dr de Vries. Analysis by SDS-PAGE (Fig 2.4.a.) showed that the protein solution was virtually free of contaminant proteins. Also, 6hisG_Lecto was recognised by an anti-SP25 specific rabbit polyclonal antiserum but not by normal rabbit serum in an immunoblott assay (Fig 2.4.b).

The immunogenicity of 6hisG_Lecto was compared to Peptide-2-KLH and FP5RsaI by administering 160 µg of each EAV antigen (160 µg of 6hisG_Lecto, 1 mg of FP5RsaI, 320 µg of Peptide-2-KLH respectively), with Carbopol as adjuvant, to 2 ponies by intramuscular injection on weeks 0, 7 and 13 and monitoring antibody responses by the Peptide-1 ELISA and the VN test. Figure

2.5 displays the serology results which show, consistent with previous experiments, good ELISA antibody responses by all ponies soon after vaccination. In contrast, two or three doses were necessary in order to induce a measurable VNAb response. Peptide-2-KLH induced low titre VNAb's only after the third dose was given. FP5RsaI vaccinated ponies sera presented low titres (0.22 and 0.45) 2 weeks after the second dose which increased further after the last boost (0.6 and 2.2). The highest titres (2.6 and 2.3) were obtained after the third vaccination with the 6hisG_Lecto protein, which rose from 1.125 and 1.05 respectively on week 7. These results indicate that 6hisG_Lecto presents neutralising epitopes more efficiently than the other antigens tested.

To test the presence of VNAb's with anti-6hisG_Lecto specificity in EAV convalescent horse sera, a panel of serum samples were depleted of anti-6hisG_Lecto antibodies and analysed by the VN test and 6hisG_Lecto ELISA before and after the antibody depletion. For this purpose Ni²⁺ NTA beads coated with 75 µg of the recombinant protein were incubated with 0.5 ml of equine sera diluted 1/10 in MEM. The serum samples included a number of EAV VNAb positive field sera, 2 samples from EAV experimentally infected ponies (C65 p.i. and 37Cp.i.) and one sample from a 6hisG_Lecto vaccinated pony collected 2 weeks after receiving the third dose (B6A p.v3). As controls, the B6Ap.v3 and 37C p.i. samples were also incubated with uncoated beads. The results displayed in Table 2.6 show that incubation with 6hisG_Lecto coated beads reduced completely the ELISA absorbance values of all samples from EAV infected animals but only partially of B6A p.v3 (6hisG_Lecto vaccinated pony), whereas incubation with uncoated beads did not reduce the anti-G_L reactivity. However, VNAb titres from control samples (B6A p.v3 and 37C p.i.) incubated with

uncoated beads showed a small decrease (0.3 and 0.4 respectively) after antiG_L antibody depletion. The VNAb titre reduction was considered significant only when this reduction in VNAb titre was twice the value of that observed for the control depleted sample 37C (i.e. 0.8). A significant decrease in VNAb titre was observed in some samples but not in others despite efficient depletion of anti-6hisG_Lecto antibodies. This suggests that 6hisG_Lecto resembles better than Pep-2-KLH (Experiment II) the tertiary protein structure of VNAb antigenic domains and that the incomplete reduction of the VNAb titre in all the samples, except B6A p.v3, is possibly due to the presence of VNAb's which bind epitopes not mimicked by 6hisG_Lecto. The fact that the VNAb titre could be reduced in sample c65 p.i., which showed low antiG_L reactivity before applying the procedure, is difficult to interpret. However, it is noted that a previous assay showed a high ELISA absorbance value for this particular sample.

The antibody response following immunisation with 6hisG_Lecto and the results of the depletion assay showing the capacity of this protein to bind VNAb's *in vitro* prompted continuing the investigation of this protein as a potential sub-unit vaccine for EAV. Specifically, VNAb induction after 6hisG_Lecto vaccination using three different doses, duration of the antibody response and protection of the immunised ponies against experimental infection were studied.

2.3.2. 6hisG_Lecto stimulates VNAb responses and provides protection against experimental infection.

2.3.2.1. Antibody responses to 6hisG_Lecto.

The ability of different doses of the prototype sub-unit vaccine to elicit VNAb was evaluated in three groups of Welsh Mountain Ponies, each group comprising

one yearling colt and three 2-year old geldings. Group A (p1, p2, p3 and p4), Group B (p5, p6, p7 and p8) and Group C (p9, p10, p11 and p12), were vaccinated initially (V1) with 6hisG_Lecto doses of 140, 70 and 35 µg respectively (administered intramuscularly with Carbopol). The results (Figure 2.7.) show that following the priming vaccination, a weak VNAb response was observed in Groups B and C but not in Group A. A second dose (V2) on week 5 (140µg for Group A, 70 µg for Group B, 35 µg for Group C) boosted the antibody response in all ponies, with the highest titres observed for individuals from Group B (VNAb titres 2.3 to 3.1). Five weeks following V2, two ponies from Group A and an unvaccinated control pony were infected with EAV (challenge 1, described in more detail below). Protection was observed despite moderate VNAb titres for both vaccinates. A third vaccine dose (V3) was administered to all ponies on week 13 (140 µg for Group A, 70 µg for Group B, 35 µg for Group C). This induced another peak in the antibody response after which the two remaining ponies from group A were challenged on week 15 (challenge 2, described below). In this case, both vaccinates displayed a high degree of protection. The remaining ponies (Groups B and C) were sampled over a period of 40 weeks to determine the duration of the antibody response. VNAb declined gradually, being still detectable in all ponies on week 27, but undetectable by week 53.

The kinetics of the anti-G_L antibody response measured by ELISA were broadly in line with the VNAb response (Fig. 2.8) although they show a slower rate of decline. In particular, the ELISA values remain at a high level for several weeks after V3. This could be due to the high sensitivity of the ELISA and the high anti-G_L specific antibody concentration of the serum samples. In order to test

whether ELISA antibody titres may decline at a similar rate to VNAb's, various dilutions (1/100, 1/400, 1/1600 and 1/6400) of sera from ponies p9, p10, p11 and p12 collected on weeks 5, 6, 8, 10, 13 and 15 were analysed. The results confirm that the ELISA antibody response show very similar kinetics to the VNAb response (Fig. 2.9).

Following determination of the kinetics of VNAb induction and decline, the remaining ponies received a fourth vaccination (V4), comprising 35 µg of 6his G_Lecto, on week 53. This dose was administered to all remaining ponies since it appeared as effective as the other higher doses used previously. Following V4, VNAb responses were monitored and a series of EAV challenge infections performed, in order to establish the protective efficacy of vaccination across a range of VNAb titres. The results of these challenges are described below.

2.3.2.2. Protection against experimental infection

Previous challenge infections in naïve ponies with LP3A virus indicated that intranasal administration of this virus (10^6 TCID₅₀) resulted in reproducible clinical signs, virus shedding from the nasopharynx and cell-associated viraemia (Hannant D, Westcott D and Castillo-Olivares J, unpublished observations).

The results of each of the six experimental challenge infections of the present study are displayed in Fig. 2.10. a – f. and summarised in Table 2.11. For each challenge, two vaccinates and one control pony were used. In all challenges, the control ponies became infected presenting a syndrome characterised by pyrexia lasting 6 to 11 days (maximum temperatures 40 – 40.5 °C), viraemia (detected for 6 or 7 days) and virus shedding from nasal secretions for 8 to 11 days. All controls seroconverted to EAV by the neutralisation test (seroconversion defined

as an increase in VNAb titre of at least fourfold). Depression, mild conjunctivitis, sub-mandibular lymph node swelling, nasal discharge and diarrhoea were observed in some ponies.

Preliminary analysis of the challenge data indicated that vaccinated ponies showed a range of clinical and virological outcomes. It is clear that for challenge 2 (Fig. 2.10. b), vaccinated ponies are strongly protected against infection, with negligible pyrexia, viraemia or virus shedding in comparison with the control pony. Even for the vaccinates with undetectable VNAb (challenge 6, 19 weeks after V4), partial protection was observed, as judged by reduced severity and duration of pyrexia and substantially reduced virus shedding (Fig. 2.10. f). Protection against infection in vaccinated ponies was quantitatively evaluated according to objective and measurable variables consistently reproduced in the control ponies, namely the intensity and duration of pyrexia, duration of viraemia, duration of virus excretion, maximum virus excretion, total virus excretion [nasal swab samples with virus concentration below the detection limit ($10^{1.75}$) were assigned a value of $10^{1.45}$ (mean of 0 and $10^{1.75}$)] and seroconversion (Table 2.11). Protection was maximum in ponies p6, p1 and p4 which had VNAb titres above 1.95 at the time of challenge. These ponies did not seroconvert to EAV, febrile reaction and viraemia were totally prevented and virus excretion was absent or limited to a single day ($<10^{1.75}$ TCID₅₀/ml nasal swab). The other vaccinated ponies displayed varying levels of protection. All ponies with a VNAb titre <1.95 seroconverted to EAV, but pyrexia duration and intensity were reduced compared with the controls, with only one vaccinate (p10) having a peak of temperature above 40 °C and only one vaccinate (p5) with pyrexia for 6 or more days. Furthermore, the vaccinates displayed a

reduction in the duration of viraemia and nasal virus excretion, and reduced virus titres from nasal swabs. Even those vaccinates with negligible VNAb titres at the time of challenge (19 weeks after V4) were partially protected, with a substantial reduction in virus shedding (over 100 fold) compared with the corresponding control pony (Table 2.12).

In order to determine the significance of protection against each of these variables statistical advice was sought from Ken H. Lakhani who performed an Analysis of Variance. The results of this analysis (Table 2.12) indicate that the 6his-G_Lecto sub-unit vaccine provided significant protection ($p < 0.01$) against EAV infection as judged by a reduction of pyrexia, cell-associated viraemia and virus shedding from the nasopharynx. The correlation between VNAb titre at the time of challenge and total virus excretion was found to be significant as the results of linear regression analysis performed by Ken H. Lakhani show (Figure 2.13.).

2.4. Discussion

The work described in this chapter represents a follow up of previous studies demonstrating the potential of G_L derived antigens to serve as sub-unit vaccine against EAV (Chirnside *et al.*, 1995a). We found that although all the antigens tested were effective at inducing anti-G_L responses as assessed by a G_L-specific ELISA, the virus neutralising capacity of the antibodies induced by the synthetic peptides was poor as compared with the VNAb induced by bacterially expressed antigens, of which the 6hisG_Lecto antigen induced the highest titres. Analysis of the nucleotide sequences of the ORF5 of a wide range of EAV isolates and of mutant viruses escaping neutralisation by G_L-specific monoclonal antibodies

identified four regions in the G_L protein where neutralising epitopes occur (Balasuriya *et al.*, 1997). Of the antigens tested in this study, the 6hisG_Lecto recombinant protein includes all four of these antigenic sites. The anti-Peptide-2 antibody depletion experiments described in the present study suggested that either epitope conformation or number of neutralising epitopes present in the antigen could play an important role in VNAb induction after vaccination. Similar experiments performed with 6his-G_L ecto indicated the presence of VNAb binding epitopes in this recombinant protein, but it was not possible with this protein to deplete completely the VN activity of post-infection sera. This, together with the fact that some VN positive sera have low reactivity to G_L as measured by Peptide-1 or 6hisG_Lecto ELISA (Nugent *et al.*, 2000) suggests that there could be additional neutralising epitopes not presented correctly by the 6hisG_Lecto or which lie outside the ectodomain itself. Previous studies have indicated that both linear and conformational epitopes are recognised by neutralising antibodies (Deregt *et al.*, 1994; Balasuriya *et al.*, 1997). It is uncertain whether the antibodies induced by the 6hisG_Lecto antigen are restricted to recognition of linear rather than conformational epitopes. It is possible that the use of protein expression procedures in eukaryotic systems to produce glycosylated 6hisG_Lecto, combined with less stringent purification methods, might improve induction of neutralising antibodies. Balasuriya *et al.* (2000) reported that when full length G_L is expressed in eukaryotic cells, co-expression of M is required for induction of neutralising antibodies in mice. Protective immunity in horses is also higher when both envelope proteins are expressed (Balasuriya *et al.*, 2002a). It is clear from our studies that the recombinant G_L ectodomain alone, expressed in bacteria, does form epitopes required for

induction of neutralising antibodies. It would be interesting to determine whether the G_L ectodomain expressed in eukaryotic systems is similarly able to elicit VNAb.

Having identified the 6hisG_Lecto protein as the antigen of choice we conducted an extensive vaccination study using 18 ponies. Three doses of the antigen were compared to provide an indication of optimum antigen dose. We found that the medium (70 µg) and low doses (35 µg) performed similarly and, unlike the high dose (140 µg), induced detectable VNAb titres after the first vaccination. All animals developed VNAb after subsequent booster vaccinations, peaking 1 to 2 weeks after each vaccination and reaching titres > 1.8 at their peak. The medium dose induced the highest VNAb titres following the second vaccination, with 3 ponies exhibiting titres > 2.5. Two preliminary challenge infections were carried out, using ponies from the high dose vaccination group, to establish whether the VNAb responses induced by vaccination were protective (described later). The results of the challenges indicated that vaccination was protective, justifying continuation of the study to monitor the duration of VNAb responses and determine levels of protection in the remaining ponies.

Following three vaccinations, the VNAb titres in the medium and low dose groups were monitored for 40 weeks. For both groups, titres peaked 2-3 weeks after V3, but then declined gradually until week 53, when neutralising activity had become undetectable in all vaccinates. However anti-G_L antibody was still present as assessed by ELISA. The sensitivity of the peptide-1 ELISA and the good correlation with VN test would allow a quick and reliable estimation of the

immunity elicited after vaccination with the 6his-G_Lecto protein. The kinetics of VNAb development of horses vaccinated with formalin inactivated virus (Fukunaga *et al.*, 1984; Fukunaga *et al.*, 1991) are similar to those observed during this study. High titres were achieved two weeks after two vaccine doses administered four weeks apart, which then progressively declined. A third dose boosted the antibody levels, which then persisted for 6 months. Similarly, studies performed with the modified live vaccine showed that after a single vaccine dose the VNAb levels were relatively low and transient (Fukunaga *et al.*, 1981; McKinnon *et al.*, 1986; Timoney *et al.*, 1988) but supplementary vaccine administration resulted in a good anamnestic response with titres maintained for 9 to 12 months.

In order to evaluate the protective capacity of the immune responses generated by the 6hisG_Lecto protein we challenged vaccinated and control ponies with LP3A virus derived from the virulent Bucyrus strain. The challenge virus was found to consistently reproduce clinical signs and virus shedding/viraemia in EAV naive ponies (as described here and unpublished observations). With the exception of pyrexia the clinical signs observed in the control ponies were mild. The relatively mild symptoms were not entirely unexpected since the virulent pleural fluid isolate of the Bucyrus strain (derived by sequential *in vivo* passage) has been shown to lose its virulence rapidly after passage in tissue culture (McCollum, 1970; McCollum *et al.*, 1961).

Until recently (Chapter 4) there were no methods to evaluate cellular immunity against EAV, but serum VNAb are considered to be a good correlate of

immunity (Fukunaga *et al.*, 1991; Fukunaga *et al.*, 1990; McCollum, 1976). In this study we have experimentally infected 18 ponies having different levels of VNAb at the time of challenge and observed a correlation between the level of protection and the pre-challenge VNAb titre. The three ponies with titres > 1.95 showed maximum protection. None of these animals seroconverted and all were negative for viraemia. No significant virus shedding was detected (nasal swab titres $< 10^{1.75}$ TCID₅₀/ml), and although two of the ponies were positive for virus shedding, this occurred on a single day in each case. It is therefore unlikely that an EAV infection had been established. These results are similar to those reported previously in horses vaccinated with formalin inactivated virus inducing VNAb titres $> 1: 320$ (equivalent to 2.3) (Fukunaga *et al.*, 1990), although in this case seroconversion to EAV after challenge was observed despite the lack of viraemia or clinical signs. Varying levels of partial protection were observed in the rest of the 6hisG_Lecto vaccinates. All ponies with intermediate and low antibody levels, even those vaccinates with undetectable serum neutralising antibodies at the time of challenge, showed a reduction of pyrexia, nasal virus shedding and viraemia in comparison to the control ponies infected at the same time. Similar results have been described by Fukunaga *et al.* (1991) in his evaluation of the efficacy of a whole virus killed vaccine, where vaccinated horses with VNAb titres $> 1: 80$ (equivalent to 1.9) showed complete clinical protection against EAV. It was interesting to observe that after challenge, pony p5 became febrile by day five and virus began to be isolated from nasal swab and peripheral blood samples collected after that day. This apparent increase in the incubation period may indicate that this animal resisted the initial challenge but became infected subsequently as a consequence of exposure to virus shed by the

control pony. 'In contact' transmission of EAV from experimentally infected to susceptible seronegative mares has been reported previously (McCollum *et al.*, 1987). This phenomenon raises the question of whether protection would have been better if controls and vaccinates had been kept separate after challenge. In our study all unvaccinated ponies excreted large amounts of virus after challenge every day for 7 to 10 days.

In this study we have assessed the protection afforded by the experimental vaccine against intranasal challenge. By analogy with previous studies we anticipate that this vaccine has the potential to protect against venereal challenge, since efficacy studies performed with an inactivated whole virus vaccine showed similar results independent of the route chosen to challenge the vaccinated animals (Fukunaga *et al.*, 1997; Fukunaga *et al.*, 1990). In the case of ponies with VNAb titres < 1.95 (the threshold for complete protection), viraemia was generally observed. Whether under such conditions there will be protection against abortion, or the establishment of persistent infection in stallions, is uncertain. However, the significant reduction in the duration and titre of virus shedding observed in vaccinates, even when VNAb titres were no longer detectable, suggests that sub-unit vaccination could provide significant protection against respiratory spread of infection for at least five months following a boost.

We have used in the present study three viruses derived from the prototype Bucyrus strain: Utrecht Bucyrus for preparation of the recombinant antigen, CVL for the VN test and LP3A for challenge. Plasmids for expression of

recombinant G_L were derived from cDNA from the Utrecht Bucyrus strain, which had been used to determine the complete genomic sequence of EAV (den Boon *et al.*, 1991). The CVL strain has been developed as a standard reagent for EAV VN tests and was therefore utilised for determination of VN Ab titres in this study. Both the above strains have been passaged in non-equine cells and were not considered suitable for use in experimental challenges. Hence the third strain (LP3A), derived from the original Bucyrus isolate by *in vivo* passage (McCollum *et al.*, 1971) followed by plaque purification and passage exclusively on equine cells (Westcott *et al.*, unpublished data), was utilised as the challenge virus. As noted previously, LP3A had been observed to produce consistent virus shedding, viraemia and pyrexia upon infection of naïve ponies. Alignment of the G_L amino acid sequences of the three viruses used is shown in Figure 2.14. Within the ectodomain region of the vaccine, two amino acid differences are observed between the vaccine sequence and the challenge virus and five amino acid changes between the vaccine sequence and the neutralisation test virus. Notably, the LP3A virus has preserved the potential N-linked glycosylation site (aa 81-83) of the original Bucyrus isolate (also found in the majority of field isolates: Stadejek *et al.*, 1999), which is lost (N₈₁-D) in the other two isolates. Despite these changes, equine sera raised against the G_L ectodomain of the Utrecht Bucyrus isolate neutralised effectively *in vitro* the CVL Bucyrus and *in vivo* the LP3A isolates. Thus, any antigenic differences that may exist between the G_L of the vaccine and the viruses used to detect VN Ab and for challenge did not prevent virus neutralisation. A single serotype of EAV is recognised and previous studies (Balasuriya *et al.*, 1997) demonstrated that four different post-infection equine sera were able to neutralise a wide range of different EAV

isolates, although at varying titres. In view of the observed sequence variation of G_L, further studies are required to evaluate the potential of G_L vaccination to protect against heterologous isolates of EAV, representative of naturally occurring field isolates.

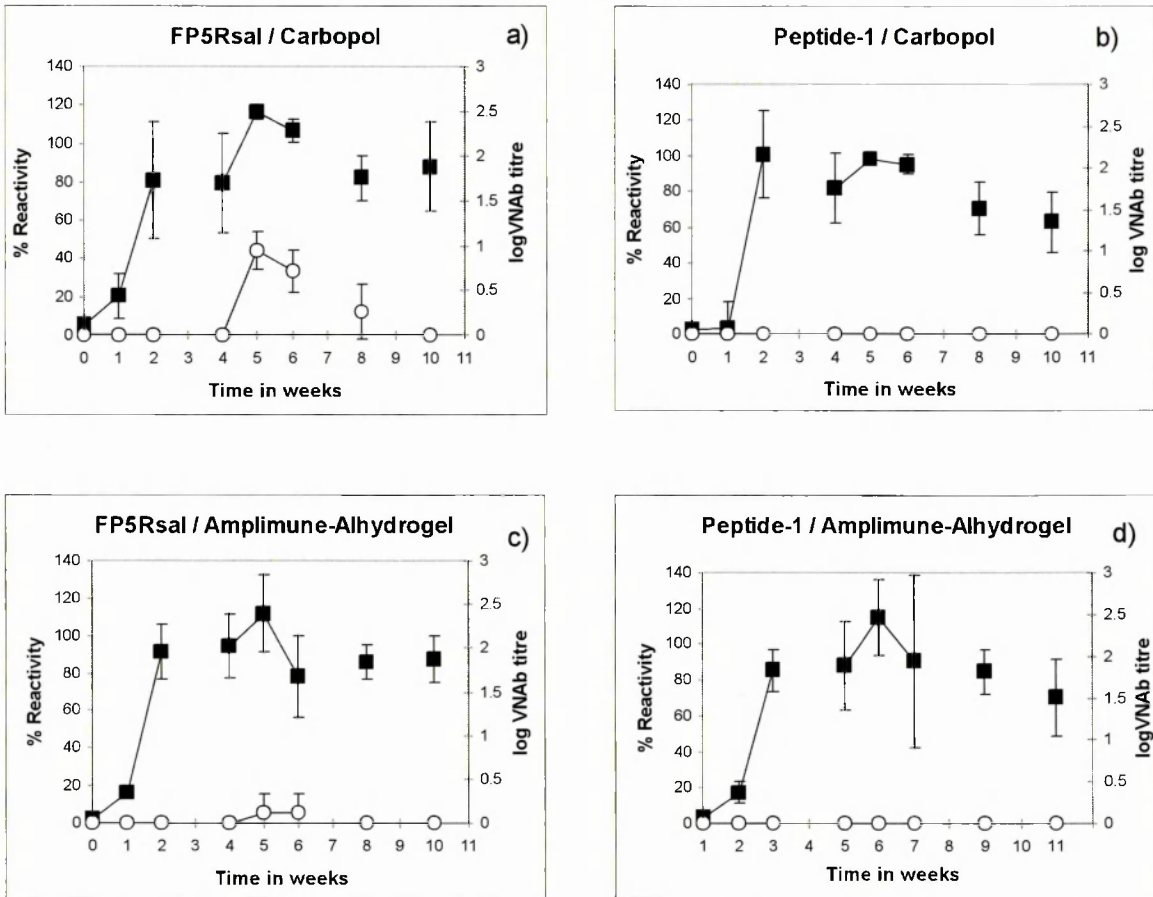
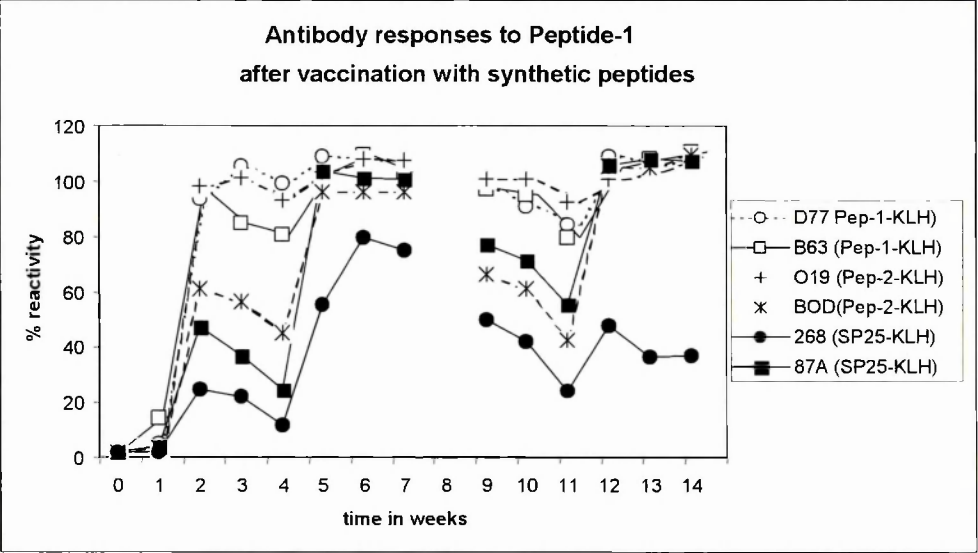


Figure 2.1. Virus Neutralising Antibody (open circles) and Peptide-1 specific antibody (closed boxes) responses of 4 groups of 4 ponies vaccinated on week 0 and week 4 with either FP5Rsal (a, c) or Peptide-1-KLH (b, d) formulated with either Carbopol (a, b) or Amplimmune-Alhydrogel (c, d).

a)



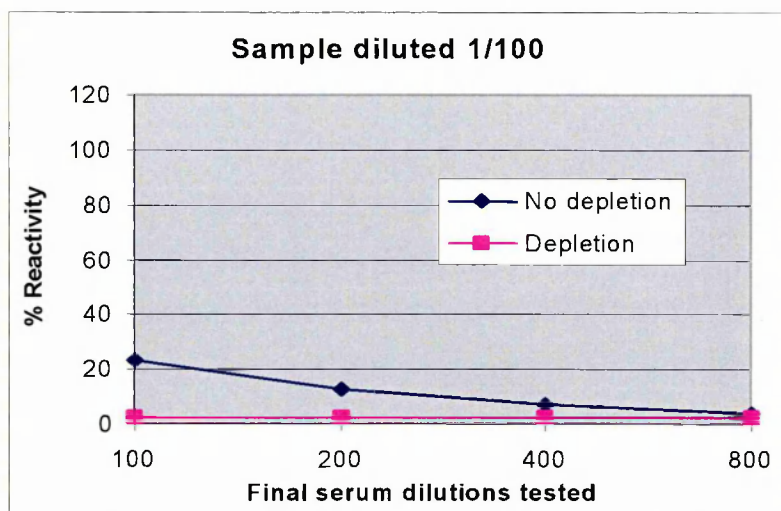
b)

VNAb responses of ponies vaccinated with Synthetic Peptides.

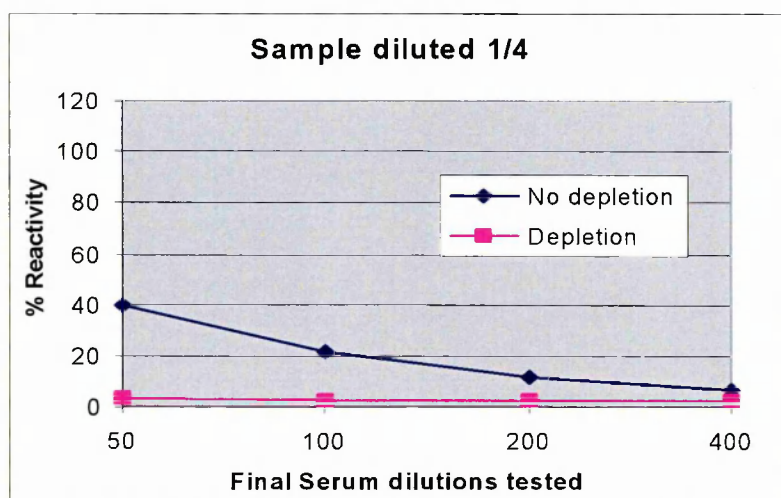
Pony/Vaccine	Time in Weeks											
	0 (V1)	1	2	3	4 (V2)	5	6	7	11(V3)	12	13	14
D77/Pep1-KLH	0	0	0	-	-	-	0.3	0	0	0.45	0.75	0.7
B63/Pep1-KLH	0	0	0	-	-	-	0	0	0	0.4	0.45	0.3
019/Pep2-KLH	0	0	0	-	-	-	0.45	0	0	1	1	0.7
BOD/Pep2-KLH	0	0	0	-	-	-	0.23	0	0	0.45	0.9	0.45
268/SP25-KLH	0	0	0	-	-	-	0	0	0	0	0	0.45
87A/SP25-KLH	0	0	0	-	-	-	0.37	0	0	0.45	0.4	0

Figure 2.2. Peptide-1 specific and VNAb responses of three groups of 2 ponies vaccinated with G_I-derived synthetic peptides formulated with the adjuvant Carbopol. Vaccinations were administered on weeks 0, 4 and 11.

a)



b)



c)

Anti-Peptide 2 Ab depletion	log VNAb titre of CM EAV pos serum	
	1/4 dilution	1/100 dilution
before depletion	2.2	2.5
after depletion	2.3	2.5

Figure 2.3. Reactivity to Peptide-1 antigen by ELISA (a and b) and VNAb titres (c) of an EAV positive horse antiserum (CM) before and after depletion of anti-Peptide-2 specific antibodies.

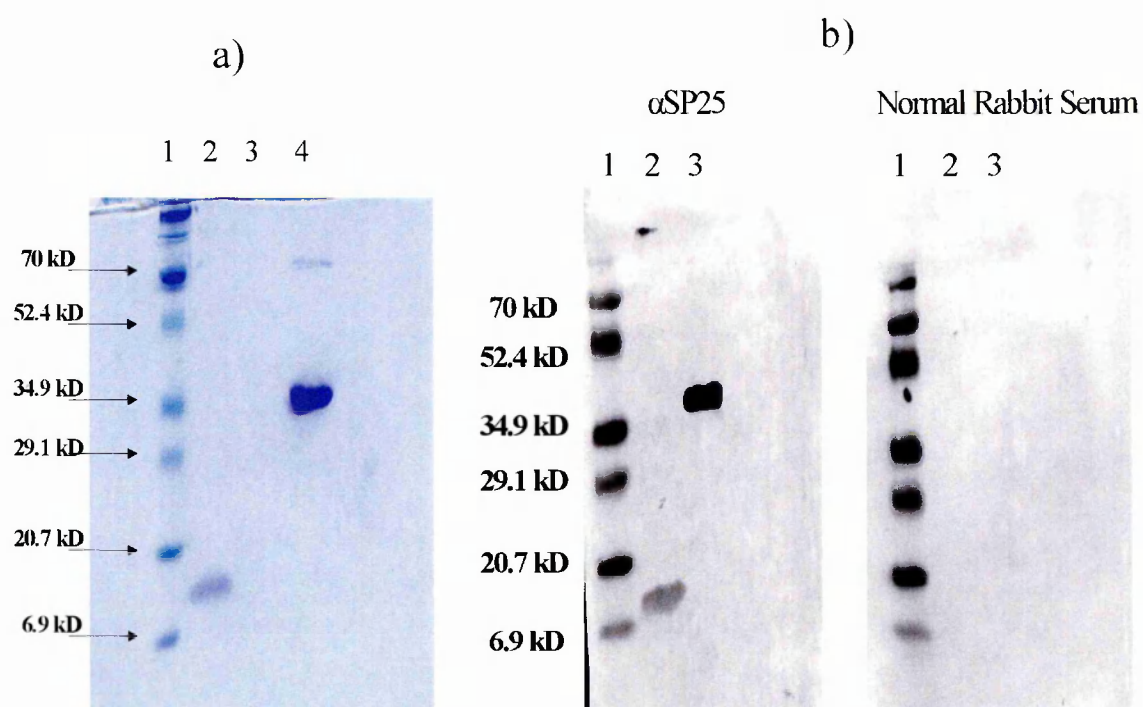
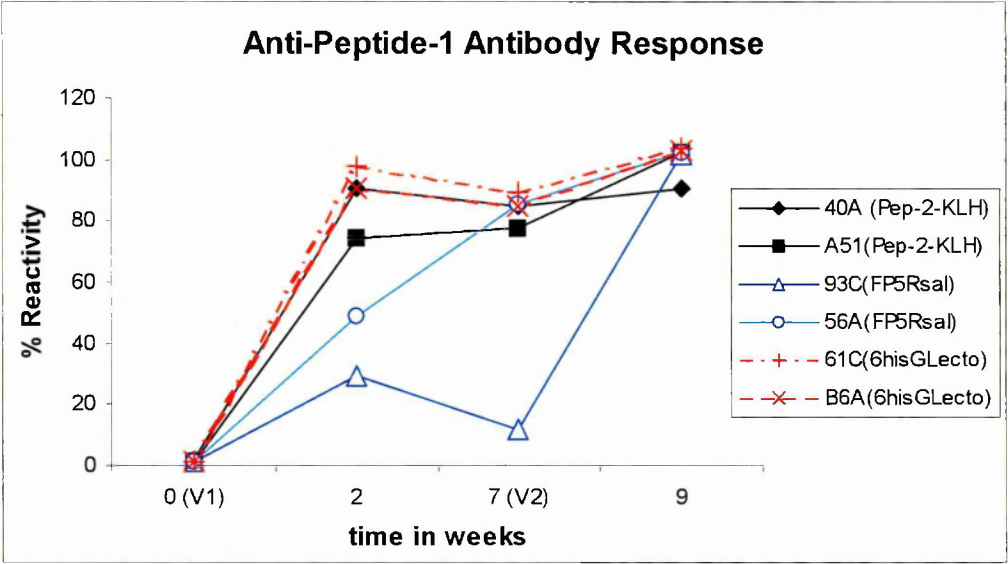


Figure 2.4. a) Electrophoretic mobility of the 6hisG_Lecto (lane 2) and FP5RsaI (lane 4) proteins on a 12.5% SDS-polyacrylamide gel. Lane 3 was empty. b) Immunoblots of 6hisG_Lecto and FP5RsaI with anti-SP25 rabbit antiserum (left panel) and normal rabbit serum (right panel). Lanes 1 in all the figures correspond to a pre-stained Molecular Weight Marker.

a)



b)

Vacc	Pep2KLH		FP5Rsal		6hisrGL	
Pony	40A	A51	93C	56A	61C	B6A
week 0 (V1)	neg	neg	neg	neg	neg	neg
week 2	neg	neg	neg	neg	neg	neg
week 7 (V2)	neg	neg	neg	neg	neg	neg
week 9	neg	neg	0.3	0.45	1.13	1.05
week 13 (V3)	neg	neg	0.45	0.5	1.6	1.3
week 15	0.9	0.45	0.6	2.2	2.6	2.3

Figure 2.5. Antibody responses of ponies immunised with Carbopol adjuvanted G_L -derived vaccines measured by the Peptide-1 ELISA and Virus Neutralisation test

		Incubation with GL coated beads						
		Previous results		ELISA O.D.		VN Ab Titre		VN Ab Titre reduction
Serum samples		ELISA O.D.	VN titre	Pre inc	Post inc	Pre inc	Post inc	
600112	1	0.3	3.1	0.5	0.1	3.2	1.8	1.4
603217	2	0.1	1.8	0.1	0.1	1.7	1.2	0.5
610356	3	0.3	2.0	0.3	0.1	2.1	1.4	0.7
506246	4	0.1	1.2	0.1	0.1	1.6	1.2	0.4
608194	5	0.1	1.5	0.1	0.1	2.1	1.5	0.6
713225	8	2.6	2.4	2.1	0.1	2.5	1.0	1.5
713459	9	2.9	2.3	2.9	0.1	2.3	1.3	1.0
710831	10	2.3	2.0	1.6	0.1	2.3	2.0	0.3
710326	11	1.3	1.9	0.6	0.1	1.8	1.1	0.7
B6A p.V3	12	2.8	2.3	3.5	1.5	2.3	<0.70	>1.6
C65 p.i.	13	1.5	1.5	0.2	0.1	2.0	1.0	1.0
37C p.i.	14	0.5	2.2	0.1	0.1	2.1	1.5	0.6
		Incubation with uncoated beads						
37C p.i.	15	0.5	2.2	0.1	0.1	2.5	2.1	0.4
B6A p.V3.	16	2.8	2.3	3.5	3.4	2.6	2.3	0.3

Table 2.6. Reactivity by the Peptide-1 ELISA and the VN test of selected horse sera previously tested positive by the AHT virology diagnostic services and of sera collected from EAVUK93 experimentally infected ponies before and after depletion of 6hisGL specific antibodies.

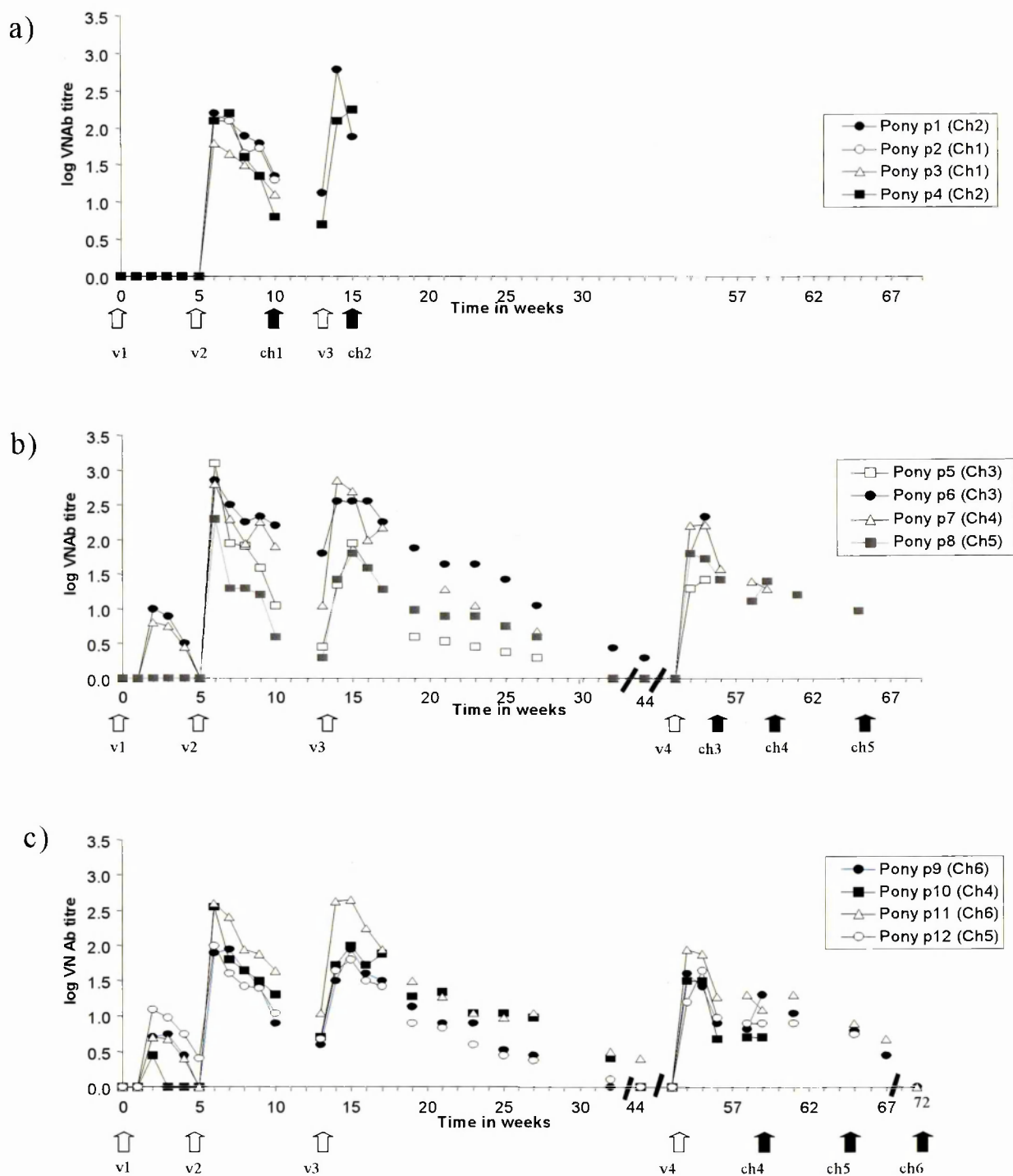


Figure 2.7. VNAb responses of ponies vaccinated with 140 (a), 70(b) or 35(c) µg of 6hisG1_ecto. Vaccines were administered (open arrows) on weeks 0, 5, 13 or 53. Selected individuals were challenged (closed arrows) at weeks 10, 15, 55, 59, 65 and 72 (Ch 1 to Ch 6 respectively).

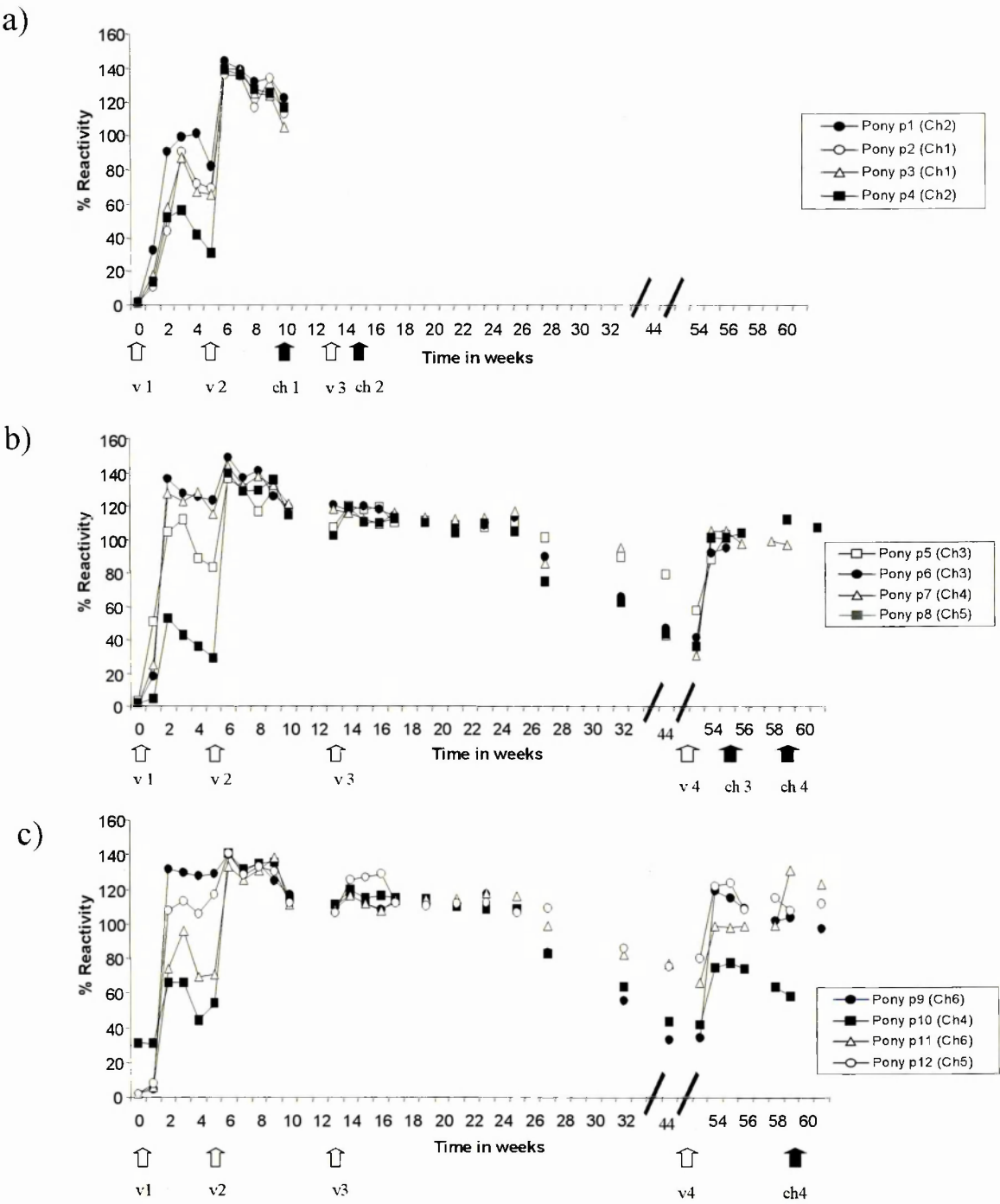


Figure 2.8. Peptide-1 specific antibody responses by ELISA of ponies vaccinated with 140 (a), 70(b) or 35(c) μg of 6hisG_Lecto. Vaccines were administered (open arrows) on weeks 0, 5, 13 or 53. Selected individuals were challenged (closed arrows) at weeks 10, 15, 55, 59, 65 and 72 (Ch1 to Ch6 respectively). Sera were analysed by ELISA only until week 62.

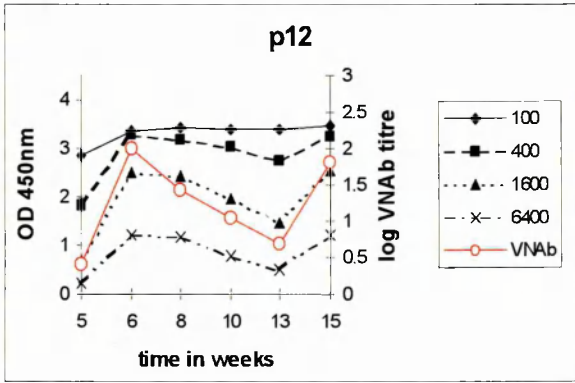
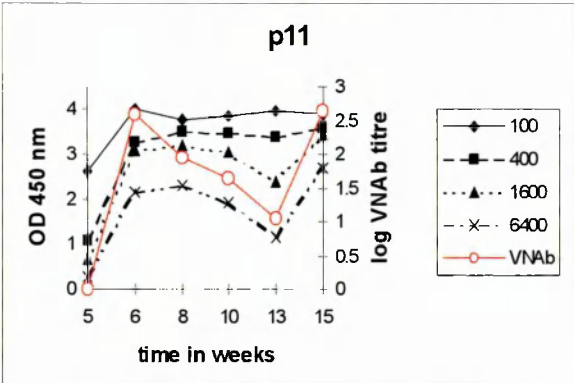
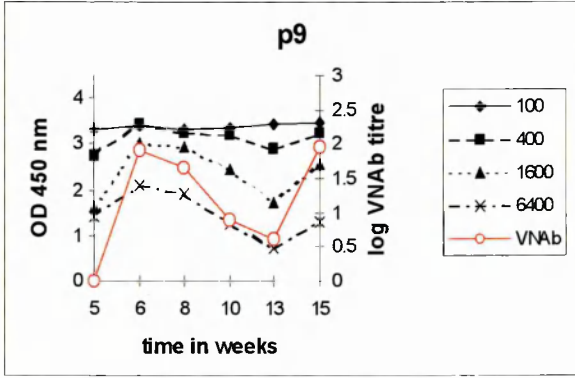
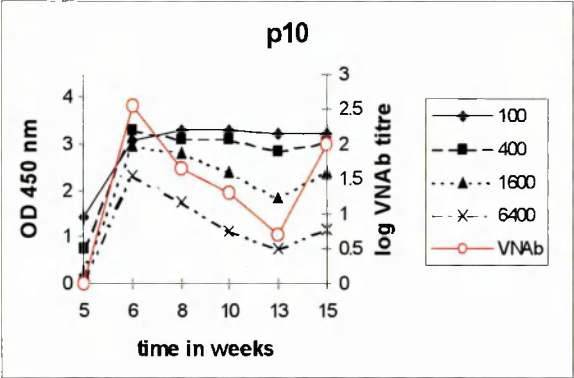


Figure 2.9. Anti-Peptide-1 specific antibody responses and VNAb titres of sera collected from ponies p9, p10, p11 and p12 on weeks 5, 6, 8, 10, 13 and 15. The sera was tested in the ELISA at 1/100, 1/400, 1/1600 and 1/6400 dilutions.

a)

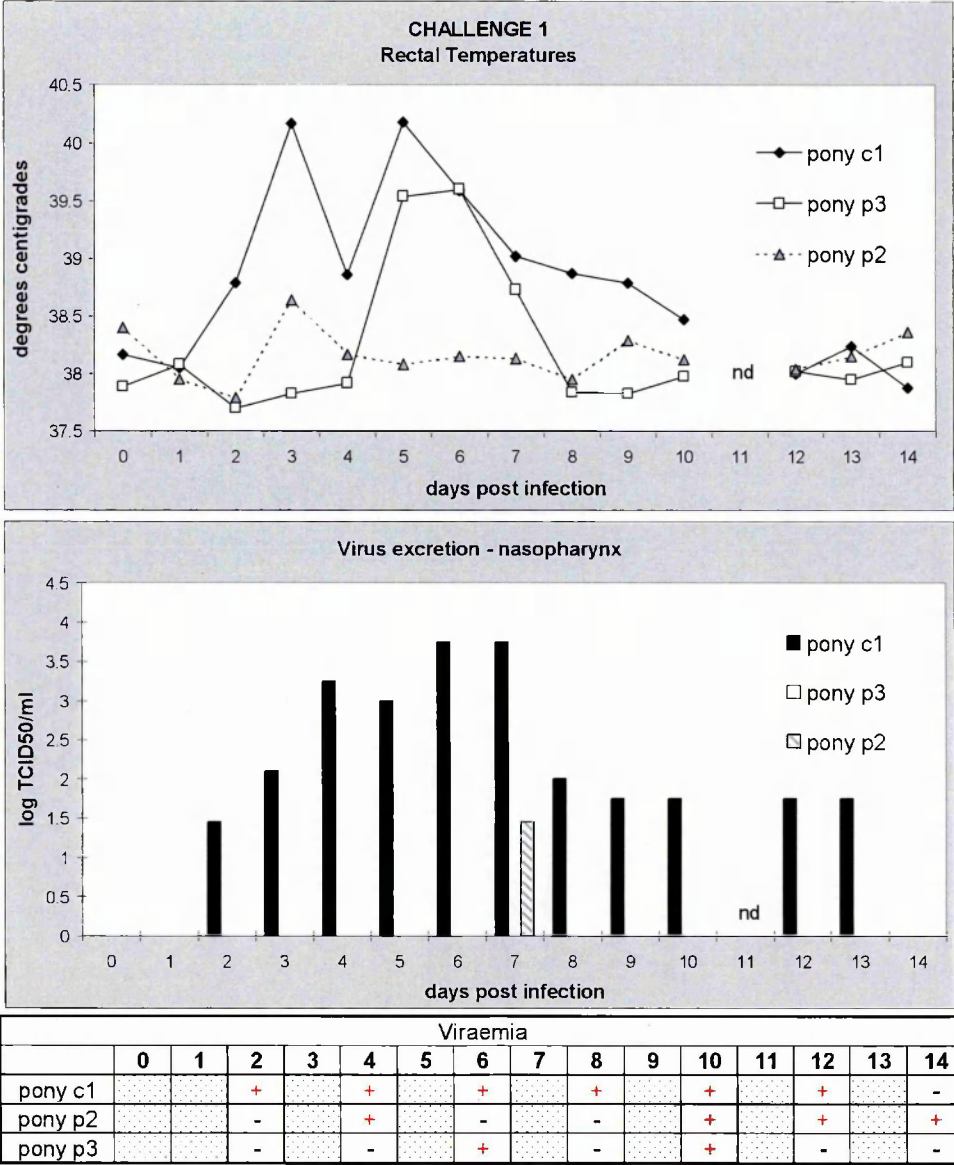
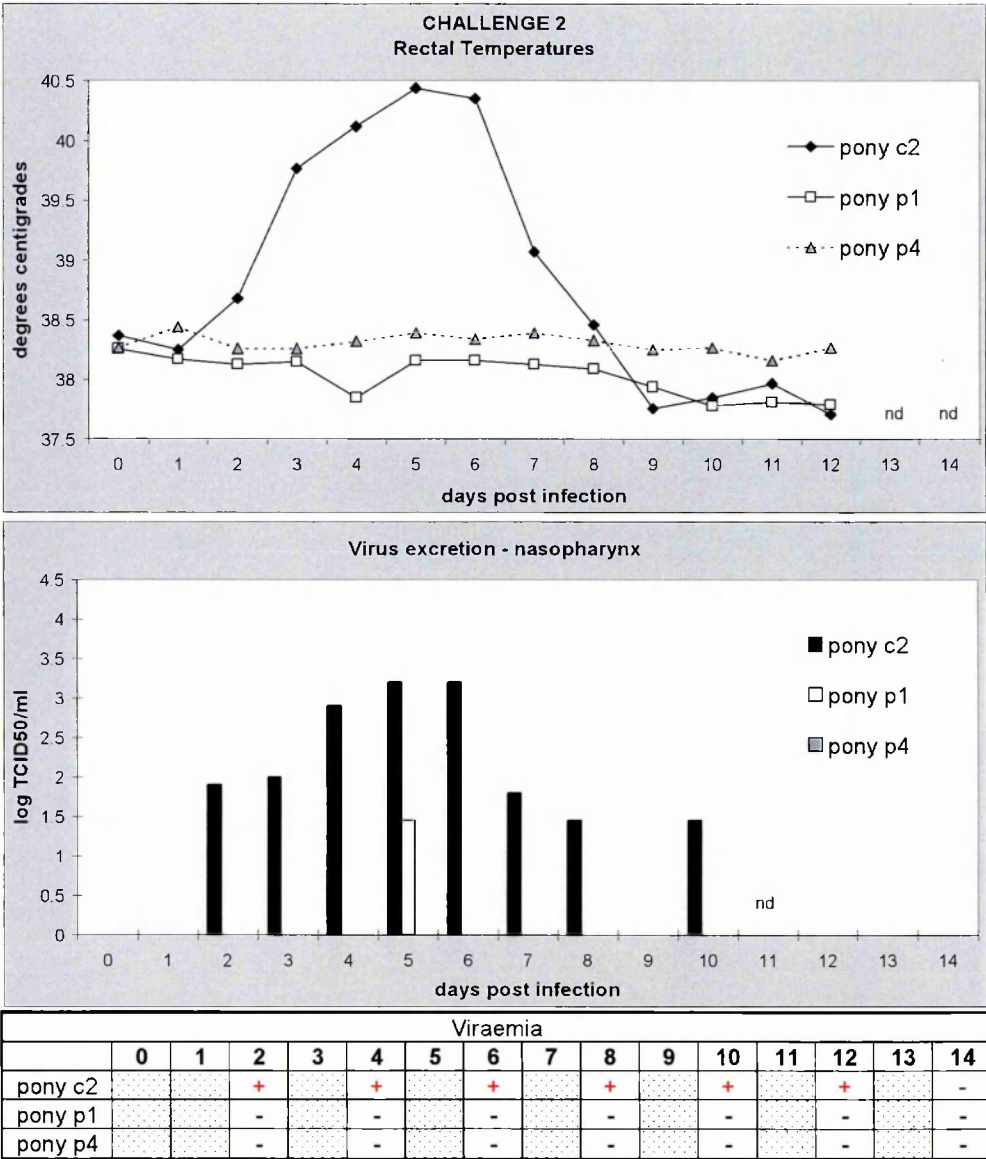
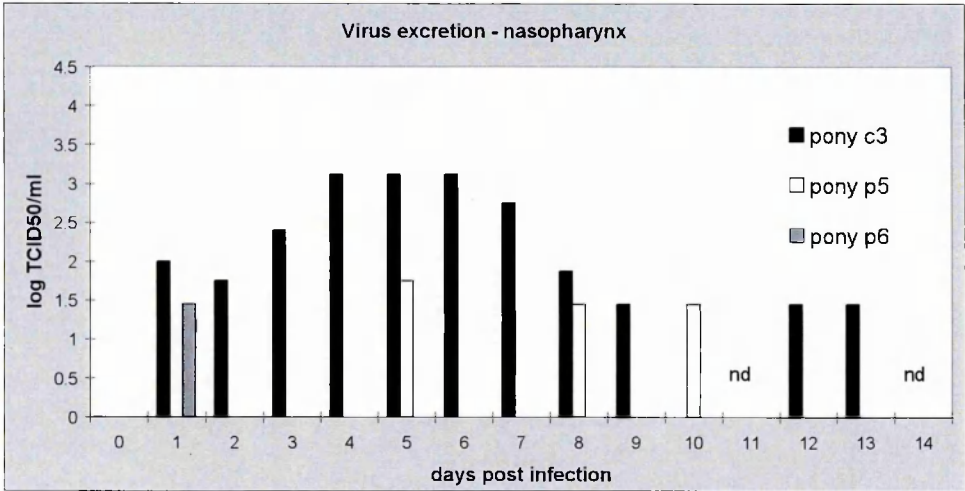
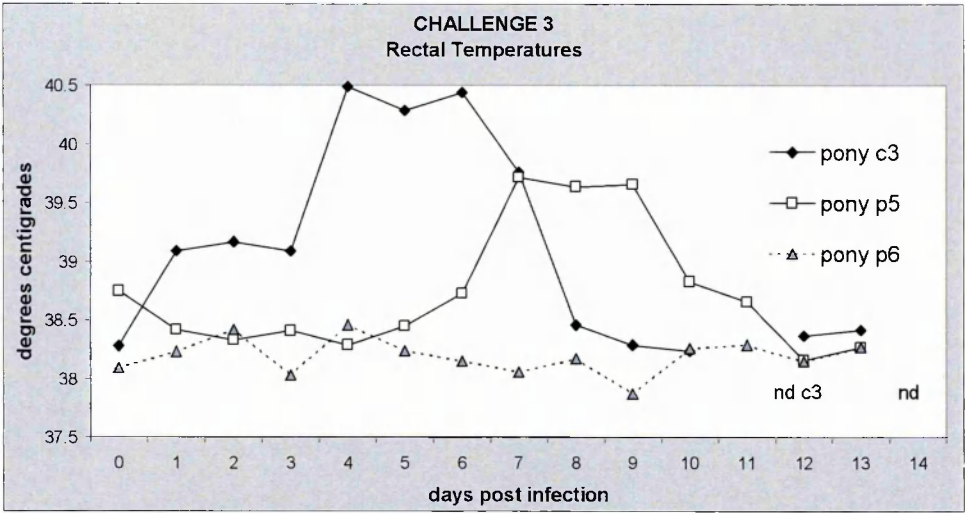


Figure 2.10. Rectal temperatures, virus excretion and viraemia of 6hisG_Lecto vaccinated (ponies p1-p12) and control (ponies c1-c6) over 14 days following exposure to virulent challenge virus LP3A. Two vaccinees and one control pony were challenged on weeks 10(a), 15(b), 55(c), 59(d), 65(e) and 72(f).

b)

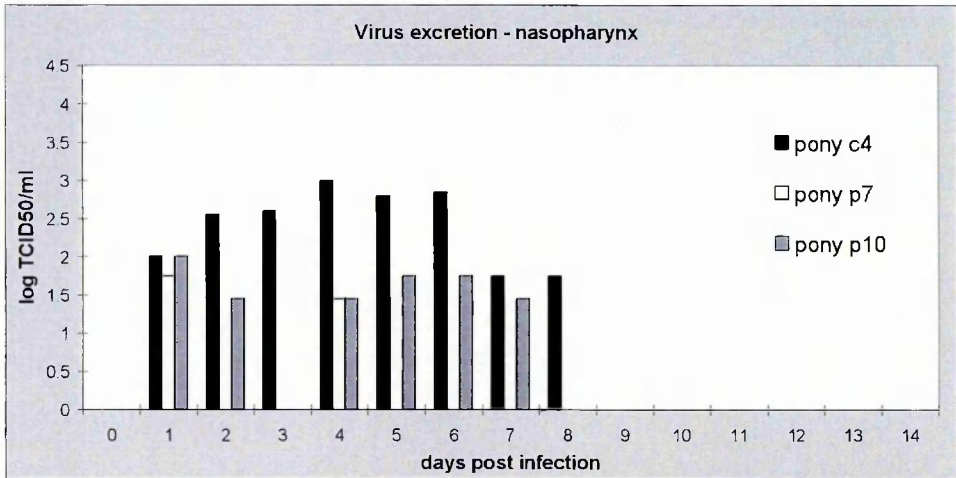
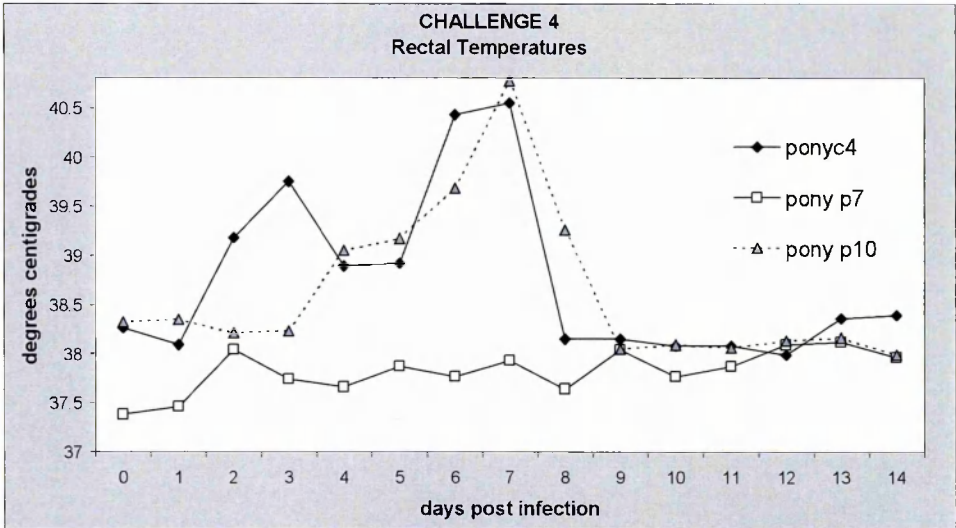


c)



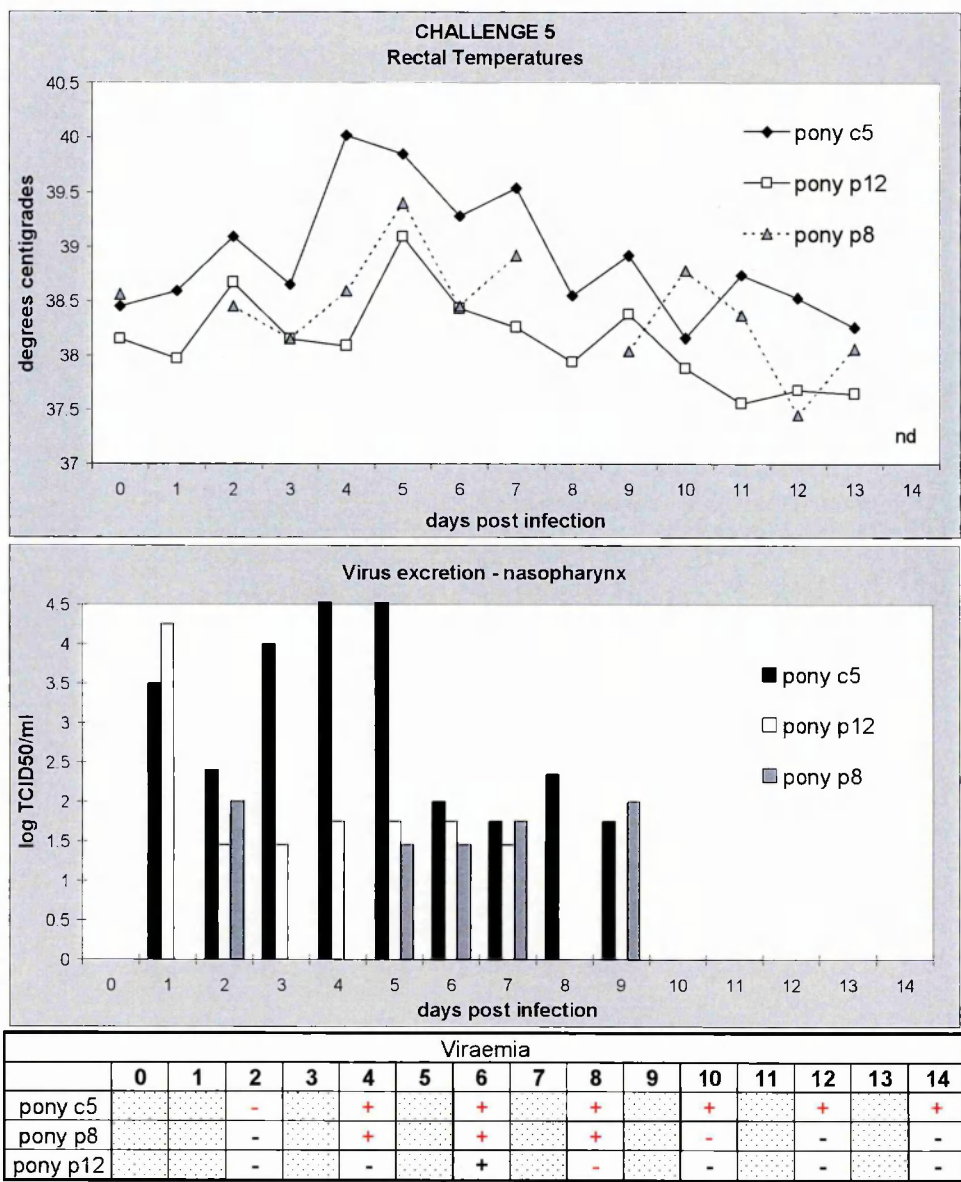
Viraemia															
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
pony c3			+		+		+		+		+		+		+
pony p5			-		-		+		+		+		+		+
pony p6			-		-		-		-		-		-		-

d)

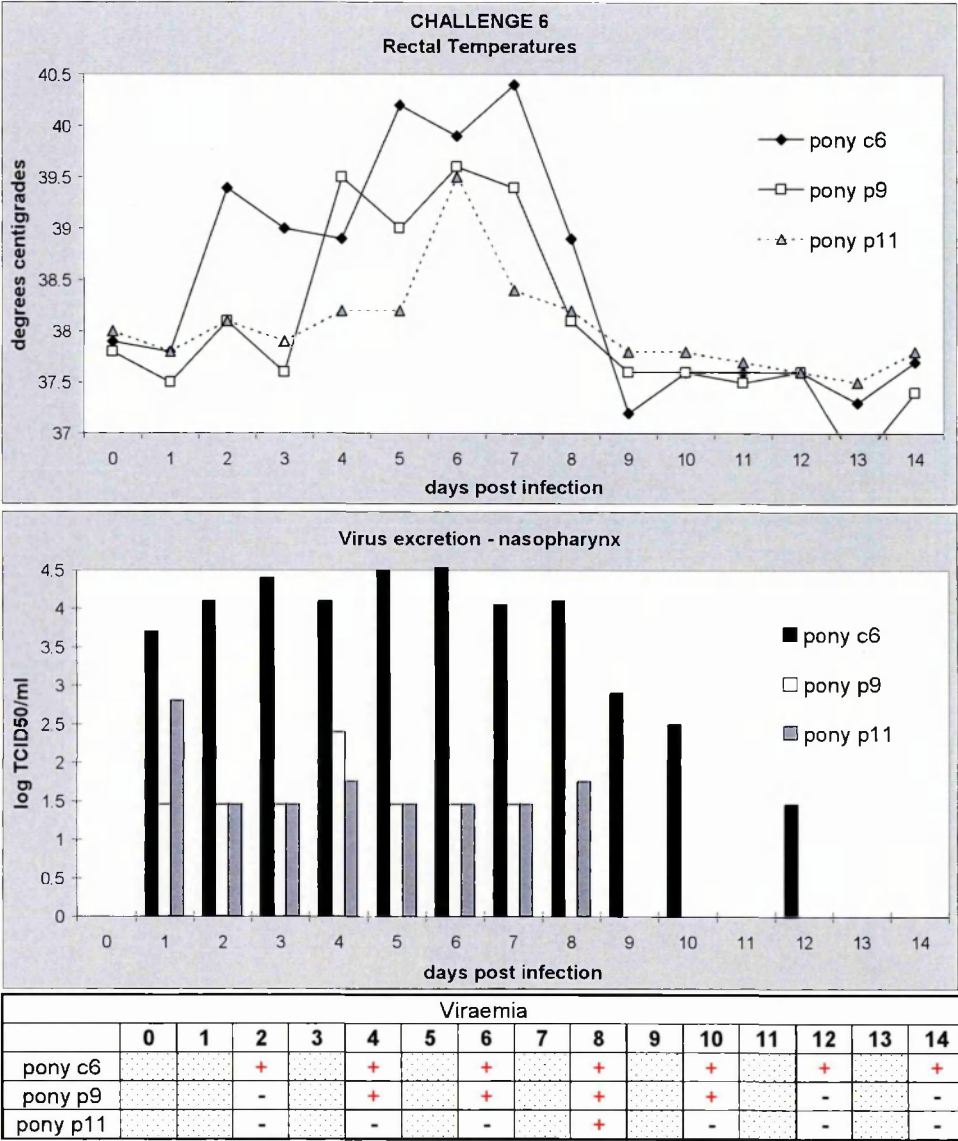


Viraemia															
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
pony c4			+		+		+		+		+		+		+
pony p7			-		-		-		-		-		-		-
pony p10			-		-		+		+		+		-		-

e)



f)



Pony ^a	VNAb titre ^b			Pyrexia ^c		Viraemia ^d	Virus shedding			Clinical Signs ^f				
	Pre	post	Seroc.	days	max Temp		Nasal swabs ^e			conj	n.d.	Ins	dep	others
p2(1)	1.30	2.30	+	1	38.64	4	1	<1.75	1.45	+	-	-	+	-
p3(1)	1.10	2.60	+	3	39.60	2	0	0	0	-	-	-	-	-
c1(1)	-	1.90	+	8	40.18	6	11	3.75	4.16	+	-	-	+	-
p4(2)	2.25	2.33	-	0	38.44	0	0	0	0	-	-	-	-	-
p1(2)	1.95	2.10	-	0	38.26	0	1	<1.75	1.45	-	-	+	-	-
c2(2)	-	2.00	+	6	40.44	6	8	3.20	3.63	+	++	+	+	-
p6(3)	2.30	2.20	-	0	38.46	0	1	<1.75	1.45	-	-	-	-	-
p5(3)	1.40	3.80	+	7	39.72	5	3	1.75	2.05	-	-	-	-	-
c3(3)	-	2.20	+	7	40.50	7	11	3.10	3.70	+	+	+	+	-
p7(4)	1.30	2.75	+	0	38.12	0	2	1.75	1.92	-	-	-	-	-
p10(4)	0.70	3.40	+	5	40.70	3	6	2.00	2.50	+	+	-	-	-
c4(4)	-	2.00	+	6	40.50	7	8	3.00	3.50	-	++	+	-	-
p8(5)	0.97	3.15	+	5	39.40	1	5	2.00	2.50	+	-	-	-	-
p12(5)	0.75	2.40	+	2	39.09	3	7	4.25	4.25	+	-	+	-	-
c5(5)	-	1.95	+	11	40.00	6	9	5.00	5.20	++	+	-	-	-
p9(6)	-	3.20	+	4	39.60	4	7	2.40	2.62	+	+	-	+	-
p11(6)	-	2.70	+	1	39.50	1	8	2.80	2.94	+	-	-	+	-
c6(6)	-	2.50	+	7	40.40	7	11	4.70	5.20	+	+	+	++	diarrhoea

Table 2.11. Clinical and virological results of experimental EAV infections performed in unvaccinated and 6hisG₁ecto vaccinated ponies. a) Pony identification number; b) Neutralising antibody titres on days 0 (pre) and day 14 post-infection (post); seroconversion (seroc.) defined as a fourfold increase in VNAb titre between pre and post titres; c) Number of days temperature was over 38.5°C and maximum temperature recorded; d) Number of days virus was isolated from blood samples; e) Number of days virus was isolated from nasal swabs, log10 maximum virus excretion values of each sample and total virus excretion (arithmetic sum of virus titres of samples collected during the first 10 days post-infection) expressed as log10; samples which were virus isolation positive, but with virus concentrations below the titration detection limit (< 10^{1.75}) were given a value of 10^{1.45} (mean of 0 and 10^{1.75}); f) Absence (-) or presence of various clinical signs of low (+) or moderate (++) severity observed after infection, namely conjunctivitis (conj), nasal discharge (n.d.), lymph node swelling (Ins), depression (dep); an isolated case of diarrhoea is also noted.

Variable	Mean \pm standard deviation		Significance	
	Control	Vaccine	F-ratio	probability
Pyrexia (days)	7.50 \pm 1.87	2.33 \pm 2.42	20.77	0.0008
Pyrexia (max. temp. °C)	40.35 \pm 0.20	39.13 \pm 0.78	11.55	0.0060
Viraemia (days)	6.50 \pm 0.55	1.92 \pm 1.83	31.54	0.0002
Virus nasal excretion (days)	8.83 \pm 0.75	3.42 \pm 3.00	36.57	<0.0001
Virus nasal excretion (max.)	3.80 \pm 0.86	1.78 \pm 1.14	28.48	0.0002
Virus nasal excretion (Total)	4.24 \pm 0.79	1.93 \pm 1.19	39.33	<0.0001

Table 2.12. Summary of statistical analysis comparing the effects of no vaccination (Control: n = 6) and vaccination (Vaccine: n = 12) for several variables indicative of severity of infection and disease following EAV infection. The means \pm standard deviation for each of the variables are shown. Statistical significance was assessed using analysis of variance (ANOVA): the F-ratio (1 and 11 degrees of freedom) to test the null hypothesis of no difference between control and vaccine treatments and the significance probability assessed by this test are shown.

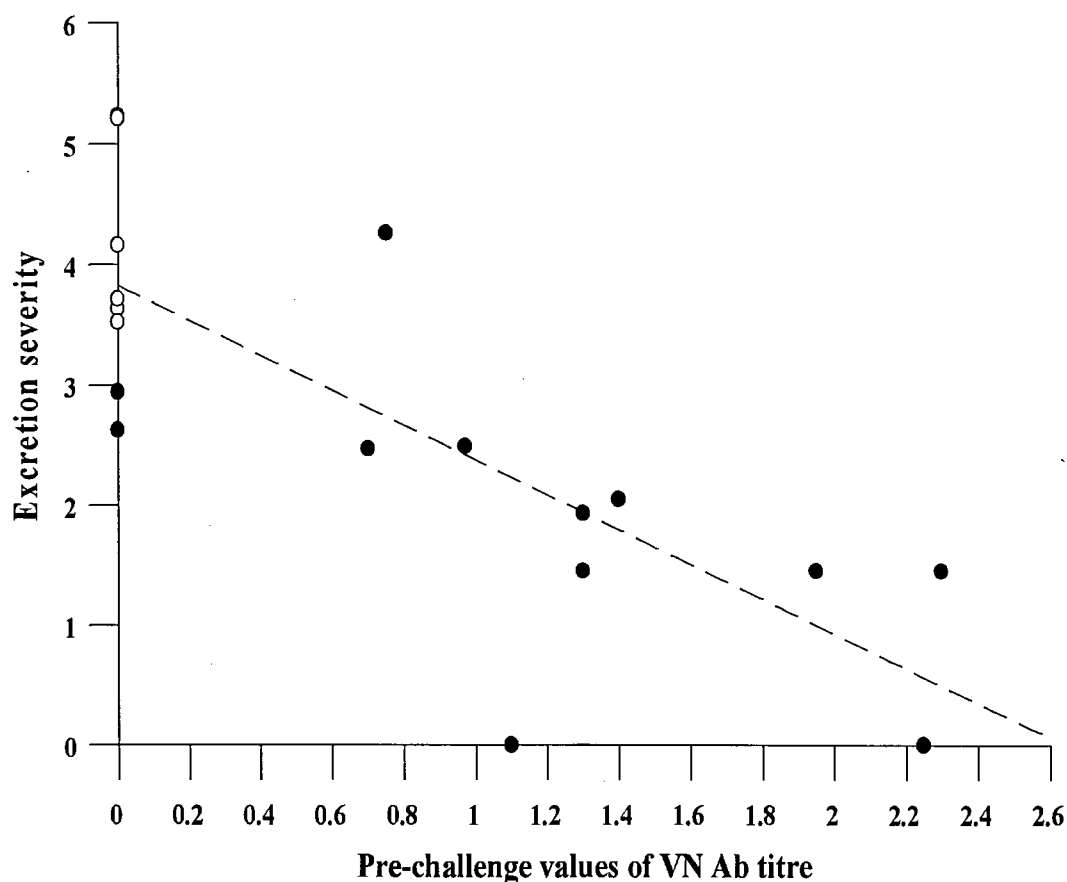


Figure 2.13. Observed relationship between total virus excretion (log of the arithmetic sum of virus titres of nasal swabs over days 1 to 10 post-challenge) and pre-challenge values of VNAb titre. Open circles show six control ponies, closed circles show twelve vaccinated ponies. The fitted linear regression model is $Y = 3.83 - 1.45 \times (\text{pre-challenge titre})$ and highly significant ($r = 0.78$, $p < 0.0001$).

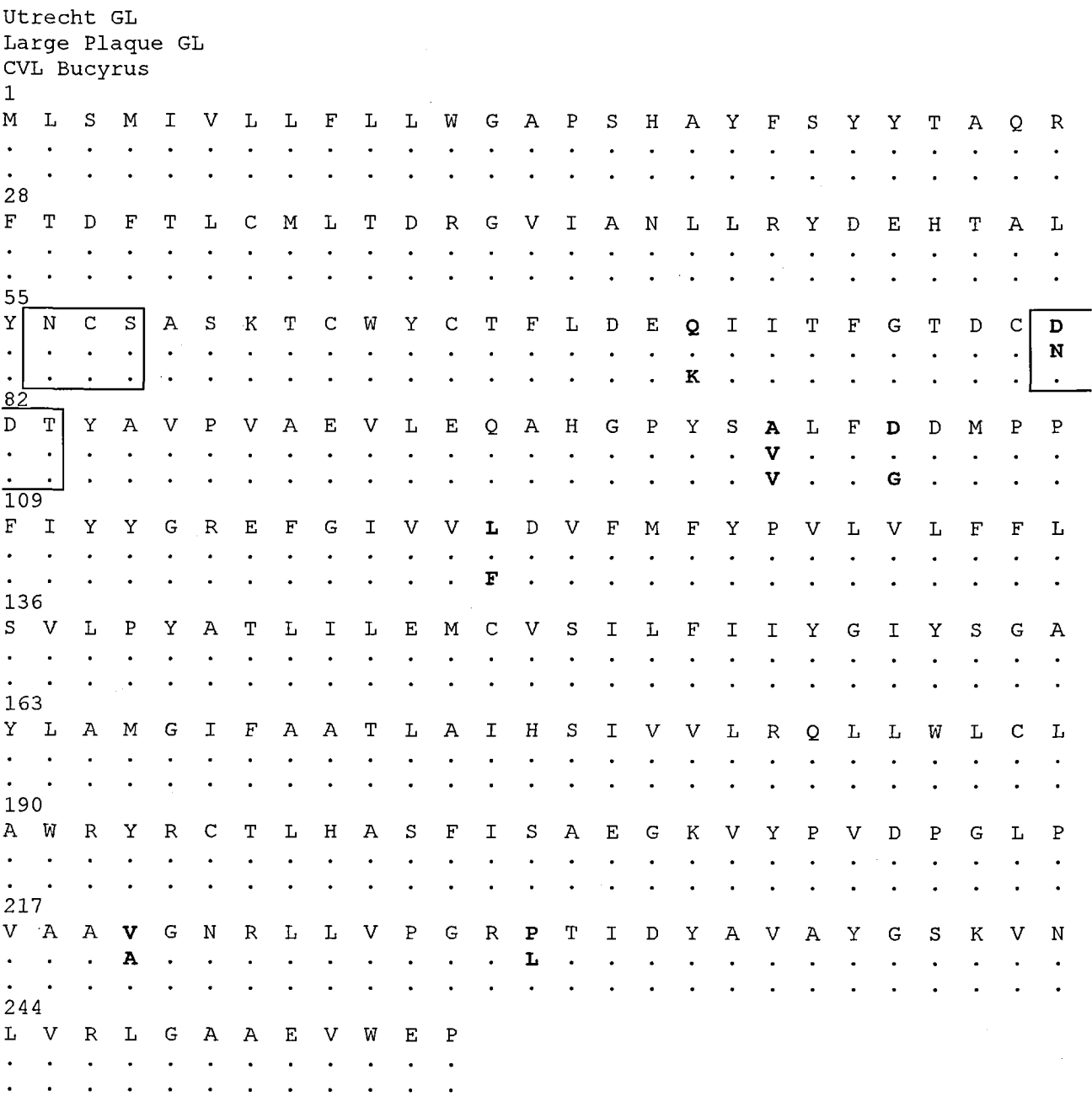


Figure 2.14 Amino acid sequence alignment of the G_L protein of EAV Bucyrus Utrecht , EAV Bucyrus CVL and EAV Bucyrus LP3A. Dots indicate the same amino acid as in the top aligned sequence. Amino acid substitutions are specified. N-Glycosylation sites are indicated in the boxes.

Chapter 3: Biological properties *in vivo* and immunogenicity of an EAV G_L deletion mutant

3.1. Introduction

Previous results demonstrated that recombinant proteins and synthetic peptides based on the EAV G_L ectodomain have the potential to serve as marker vaccines for EAV. The 6hisG_Lecto protein, like most sub-unit vaccines, is an inactive single component stimulating an effective antibody response with restricted or limited antigen specificity, which makes differential diagnosis of infection possible through detection of antibodies reactive to any of the rest of the virus antigens. In the case of EAV, prototype diagnostic procedures based on N or M antigens have been developed. The VNAb responses elicited after 6hisG_Lecto vaccination are strong and conferred different degrees of clinical protection and reduction of virus excretion and viraemia relative to prechallenge VNAb titres. However the duration of the VNAb response is short as with other sub-unit vaccines and, although cell-mediated cytotoxic responses have not been measured, it is unlikely that they were stimulated with the G_L sub-unit vaccine. Another strategy for generation of an EAV marker vaccine is explored by using a genetically modified EAV virus, made through manipulations of the full length cDNA clone of EAV.

The generation of cDNA full length clones of RNA viruses has enabled the manipulation of viruses genomes to insert or delete DNA fragments representing genes or part of them to modify biological properties such as tissue tropism, antigenicity or replication, therefore facilitating the design of live marker virus vaccines. This approach for marker vaccination offers the advantages of more

complete immune responses and longer duration of immunity. However, the requirements for demonstration of safety and attenuation are higher than those for killed or sub-unit vaccines. In the case of EAV, full-length clones of EAV have been constructed and manipulated to study fundamental aspects of EAV biology (van Dinten, *et al.*, 1997; de Vries *et al.*, 2000, 2001; Molenkamp *et al.*, 2000; Dobbe *et al.*, 2001). These manipulations included the production of single point mutations, deletions of various sizes or insertions of foreign DNA.

The principle of using a live marker vaccine for EAV using a deletion mutant virus generated through manipulation of the full-length clone of EAV is explored next. This work was performed in collaboration with the Department of Infectious Diseases and Immunology of the University of Utrecht under the direction of Professor Peter Rottier. This group performed all the molecular cloning work and generated the recombinant virus. Some of its *in vivo* properties and immunogenicity have been examined to test whether this virus could be used as a live marker vaccine.

The genetically modified virus used in our experiments, denoted as EAV-G_LΔ, was created through manipulation of the plasmid pEAN515. This plasmid is a derivative of the EAV infectious cDNA clone pEAV030-*Bg*/II KO (de Vries *et al.*, 2000; van Dinten *et al.*, 1997), in which the cryptic transcription termination signal at position 8941-8956 has been removed by site directed mutagenesis while simultaneously creating a *MscI* site (mutations: A8941C, T8945A, C8946G, T8947C, T8950C, and G8956C). To construct pEANG_LΔ, a PCR product was synthesized using the oligonucleotides 983 (5'-GAGAATTCACGGCCATACCAACAGGTTTTACTGGCGGAAC-3') and 984 (5'-CTTGCGTGCAAGTGGTTG-3') and pEAN515 as the DNA template. The

resulting PCR fragment (724 nt) was treated with *Bgl*II and *Eco*RI, and the 638 bp digestion product was cloned into *Bgl*II + *Eco*RI digested pEAN515 and sequenced. EAV RNA was transcribed *in vitro* from pEANG_LΔ, and BHK-21 C13 cells were transfected with the synthetic RNAs by electroporation essentially as described by de Vries *et al.*, (2000). The resulting EAV-G_LΔ virus presents a deletion of 138 nucleotides of the ORF5, encoding amino acids 66 to 112 of the putative ectodomain of EAVG_L (Fig. 3.1). This virus was constructed considering: a) that an EAV mutant virus presenting this deletion retained its infectivity *in vitro* (Balasuriya *et al.*, 1997) and although it escaped neutralisation by murine monoclonal antibodies it was effectively neutralised by equine polyclonal EAV specific antisera; b) that a full-length cDNA clone derived virus, EAV030H, was infectious, avirulent and genetically stable in horses (Balasuriya *et al.*, 1999b) and c) that the Peptide 1 antigen (aa 81- 106), which forms the basis of an already developed diagnostic ELISA test (Nugent *et al.*, 2000), is contained within the deleted sequence, theoretically permitting the serological discrimination of vaccinated from infected animals. It is difficult to predict however, how the absence of G_L neutralising epitopes, contained within the deleted G_L fragment, will affect the capacity of the deletion mutant virus to induce VNAb or protective immunity in ponies. To study the replication features of this virus *in vivo* and the protective efficacy and antigenic specificity of the immune responses stimulated, two ponies were infected with EAV-G_LΔ and six weeks later challenged, together with two EAV seronegative ponies, with the virulent EAV LP3A+ strain.

3.2. Materials and Methods.

3.2.1. Cells

Primary equine embryonic lung cells (EELs, fibroblastoid) were obtained from an established in-house cell line prepared from an aborted foetus. This cell line was tested to be free of mycoplasma or equine viruses. The cells were maintained in minimum essential medium Eagle with Earle's salts (Sigma, M2279) supplemented with 100 IU of penicillin per ml, 10 µg of streptomycin per ml, non-essential amino acids, L-glutamine to a final concentration of 2 mM and 10% heat inactivated foetal bovine serum (10% MEM). The continuous cell line RK-13 was used for the virus neutralisation test.

3.2.2. Viruses

The EAV LP3A⁺ virus stock, a single passage derivative in EEL cells of LP3A was used for challenge infection of ponies as well as for serological testing together with the Bucyrus CVL EAV strain (Edwards *et al.*, 1998). These two viruses differ at some positions in the G_L coding sequence (Fig. 2.14) but show a high degree of antigenic cross-reactivity.

The G_L deletion mutant EAV-G_LΔ was obtained from Dr Roeland Wieringa in Professor Rottier's laboratory. This virus was generated through manipulations of the cDNA infectious clone of EAV as indicated above. This virus stock was raised in BHK-21 cells.

3.2.3. RT-PCR

RNA was purified from tissue culture fluid of infected EEL cells or RK13 cells using the High Pure PCR Template Prep. Kit (Roche Molecular Biologicals)

following the manufacturer's instructions. Briefly, 200 µl of tissue culture fluid were mixed with 200 µl of 'Binding Buffer' (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, 20% Triton-X-100, pH 4.4) and 40 µl of Proteinase K and incubated for 10 min at 72°C. After the addition of 100 µl isopropanol, the viral RNA was bound to a glass fiber filter and washed twice with 500 µl of washing buffer (20 mM NaCl, 2 mM Tris-HCl, 80% ethanol, pH 7.5). Finally the RNA was eluted with 200 µl of 10 mM Tris-HCl, pH 8.5. As a control, RNA was similarly purified from culture fluid of RK13 cells infected with the CVL Bucyrus strain of EAV. Reverse transcription of viral RNA and subsequent amplification of cDNA of EAV ORF5 were performed in one step reaction using the *rTth* DNA Polymerase and EZ Buffer (Perkin Elmer) in the presence of manganese acetate $Mn(OAc)_2$ with primers JV1 (TTACGACTGGTACGTTGGG) and JV2 (GATACTCACCTAAAATCCCG). Purified viral RNA (5 µl) was included in a 50 µl PCR reaction containing 300 µM of each dNTP's, 0.1 units/µl of the enzyme, 0.4 µM of each primer and 2.5 mM $Mn(OAc)_2$ diluted in EZ buffer. Virus present in blood from infected ponies was checked similarly for its ORF5 deletion by RT-PCR of tissue culture fluid from infected RK13 cells.

3.2.4. Vaccination and challenge infections of ponies.

The EAV-GLΔ inoculum was prepared by infecting EELs at an m.o.i. of approximately 0.002 with BHK-21-derived virus and incubation for 72 h at 37°C. The tissue culture fluid was then harvested, freeze-thawed, cell debris was removed by centrifugation and aliquots of the supernatant were stored at -70°C.

Procedures for EAV inoculation of ponies have been described previously (Chapter 2). Two year-old castrated male Welsh Mountain Ponies (5062, 697b, 7b69 and 07d41), seronegative to EAV, were used in this study. Ponies 7b69 and 07d41 were stabled in a contained environment facility and inoculated with 10^6 TCID₅₀ of EAV-G_LΔ by the intranasopharyngeal route and monitored closely for 28 days. Nasopharyngeal swabs, heparinized blood and serum samples were collected at regular intervals and analyzed for virus and antibody as described below. After infectious virus could no longer be detected the ponies were released from containment to an isolated barn. Forty-six days after the immunisation ponies 7b69 and 07d41 and EAV seronegative ponies 5062 and 697b were inoculated with $10^{6.1}$ TCID₅₀ of EAV strain LP3A+. All four ponies were clinically inspected twice a day and serum, heparinized blood and nasopharyngeal swabs collected at regular intervals until the end of the study. The ponies were released to a third isolated barn when they were regarded as non-infectious, prior to then being transferred 2 weeks later to an isolated paddock for 4 weeks before they were allowed to have contact with any other equines.

3.2.5. *Virus isolation and serology.*

Virus isolation from blood and nasopharyngeal samples and virus neutralising antibody assays were conducted as previously described (Chapter 2). The virus neutralization test was performed using three different EAV strains: LP3A+, Bucyrus CVL and EAV-G_LΔ. In addition to the serum samples collected from the ponies used in this study a panel of EAV-specific horse antisera collected from various experimental infections conducted at the AHT with various EAV

isolates was used. Antibody responses to two G_L derived antigens were analysed by ELISA following the procedures described by Nugent *et al* (2000) with minor modifications. The antigens chosen were Peptide-1 (aa 81-106), a region deleted in EAV-G_LΔ, and 6hisG_Lecto recombinant protein comprising the complete G_L ectodomain (residues 18-122).

3.3. Results

3.3.1. Preparation of an EAV-G_LΔ virus stock for intranasal administration to ponies.

The intranasal route was chosen for immunisation / infection of the ponies with the deletion mutant virus, a route which has been used successfully over the years with various EAV stocks prepared in primary equine embryonic lung cells (EEL's). Since the recombinant virus EAV-G_LΔ supplied to us by our colleagues in Utrecht was generated in non-equine cells, this virus was passaged once in EEL's in order to prepare a stock for immunisation. Monolayers of EEL's were infected with EAV-G_LΔ at an m.o.i. of approximately 0.002 and incubated for 72 h at 37°C. At this point CPE was widespread and 80% of the cell sheet was destroyed. The tissue culture fluid was then harvested, freeze-thawed, cell debris was removed by centrifugation and aliquots of the supernatant were stored at -70°C. The titre in EEL's of the new virus stock was 10^{6.3} TCID₅₀ / ml, similar to other virus stocks of EAV used successfully for intranasal infection of ponies.

The EAV-G_LΔ virus stock was analysed by RT / PCR, using EAV specific primers flanking ORF 5 to confirm that it comprised virus with a G_L deletion.

As shown in Figure 3.3, the EAV_{G_L}Δ RT / PCR product is smaller than the wild type ORF 5 product from the CVL Bucyrus strain.

Growth curves of the parental EAV-G_LΔ virus in BHK-21 cells, performed in Utrecht (not shown) and of EAV-G_LΔ in EEL cells, performed at the AHT by Miss Emma Weston (Fig 3.2) indicate that the recombinants possess a slower replication rate than wild type EAV viruses.

3.3.2. Clinical, virological and serological responses to EAV-G_LΔ virus inoculation.

Once a suitable virus stock for immunisation was obtained and the deletion of the G_L fragment confirmed, an experimental infection of two EAV seronegative ponies was conducted to study the virus replication features *in vivo*, clinical signs and immunogenicity induced by EAV-G_LΔ.

Ponies 7b69 and 07d41 were inoculated with 1ml of EAV-G_LΔ by the intranasopharyngeal route, as indicated in the materials and methods section, and monitored for clinical signs for a period of two weeks. Nasopharyngeal swabs and blood samples were collected regularly for virus isolation until three consecutive samples gave a negative result.

Infection with EAV-G_LΔ did not induce clinical signs in ponies 7b69 and 07d41. However, cell associated viraemia and nasopharyngeal virus excretion were observed in both ponies (Table 3.4.). Viraemia was first detected on days 4 and 6 from ponies 7b69 and 07d41 respectively and lasted until day 14 in both animals (viraemia was not detected on days 16, 19 and 21, data not shown). Virus excretion from the upper respiratory tract occurred between days 1 and 7 but the

infectivity was below $10^{1.5}$ TCID₅₀/ml except for the sample collected on day 5 from 07d41 ($10^{1.5}$ TCID₅₀/ml). This contrasts with high titres obtained in nasal swab extracts from ponies infected with the wild type virus LP3A (Chapter 2). RT / PCR analysis of viral RNA extracted from the virus isolated from a blood sample collected from pony 7b69 on day 4 post-inoculation confirmed that the recovered virus carried the expected internal deletion of ORF5 (Fig. 3.3). Both ponies developed serum VNAb's against the immunising virus reaching high titres by day 21 post-inoculation. In contrast, the serum VNAb titres against two wild type EAV strains, LP3A+ and CVL Bucyrus, measured on the same day after infection were 2 log₁₀ lower. The antigenic specificity of the antibody responses was further characterised and described below in section 3.3.4.

3.3.3. Clinical, virological and serological responses to EAV LP3A+ challenge

To evaluate the immunity against EAV infection provided by previous exposure to EAV-G_LΔ, ponies 7b69 and 07d41, together with two EAV seronegative controls (ponies 697b and 5062), were infected with $10^{6.1}$ TCID₅₀ of the virulent LP3A+ virus by the intranasopharyngeal route, and clinical signs, virus excretion and duration of viraemia compared between both groups of animals (Fig 3.5).

Both control ponies developed evident clinical signs of EAV which included pyrexia, depression, anorexia, conjunctivitis, nasal discharge and ataxia in contrast to ponies 07d41 and 7b69 which remained asymptomatic during the study period. Control ponies presented cell-associated viraemia lasting up to day 21 post-infection whereas virus was detected only on day 8 from both vaccinated ponies. Virus was excreted from the upper respiratory tract from day 1 to day 12

in the case of 697b or day 1 to day 9 in the case of pony 5062. For the controls, virus infectivity of the nasal swab extracts was high ($>10^3$ TCID₅₀/ml) between days 2 to 9 (697b) or 2 to 7 (5062), reaching maximum levels ($>10^5$ TCID₅₀/ml) on days 4 and 5 (5062) or 5 and 6 (697b). In contrast, virus was only isolated on one or two occasions from 7b69 and 07d41 respectively, and the nasal swab extract infectivity was $<10^{1.5}$ TCID₅₀.

3.3.4. Serology

The results of the analysis of the antibody responses after EAV-G_LΔ infection and LP3A+ challenge are displayed in figures 3.6 and 3.7. After inoculation with the deletion mutant virus, ponies 7b69 and 07d41 developed a low titre VNAb response against the wild type EAV strains, Bucyrus CVL and LP3A+. Thus, by day 14 post-infection, VNAb's could only be detected in pony 07d41 against the LP3A+ strain. These VNAb titres against the wild type EAV strains increased slightly until the day of the challenge on day 46 when the titres for the two viruses were detectable in both ponies. Interestingly, the VNAb responses were stronger against EAV-G_LΔ, reaching titres of 1.35 and 0.8 by day 14, and 2.2 and 2.8 on day 21 post-infection (for ponies 7b69 and 07d41 respectively). At the time of challenge, VNAb titres for both immunised ponies were at least ten times higher against EAV-G_LΔ than against the wild type strains, suggesting that the deletion mutant virus differs antigenically from wild type EAV.

After challenge, both immunised ponies responded with a rapid increase in neutralising antibodies to all three viruses, with titres being generally higher against EAV-G_LΔ. The control ponies showed a typical VNAb response after EAV infection against both wild type viruses, with titres detectable by day 6 and

increasing rapidly to reach high values by day 14. In contrast, the titres against the EAV-G_LΔ virus were very low, which is consistent with the antigenic difference between the viruses.

Examination of the antibody responses to the G_L ectodomain derived antigens by ELISA (Fig. 3.7) showed an undetectable response to the Peptide 1 antigen (aa 81-106) by ponies 7b69 and 07d41 after immunisation with the deletion mutant virus, consistent with the absence of this antigenic peptide from the EAV-G_LΔ virion. A weak reaction to this antigen was observed with serum collected from pony 7b69 collected 14 days after experimental infection with LP3A⁺. In contrast, both EAV-G_LΔ immunised ponies developed an antibody response to 6hisG_Lecto (aa 18-122) which was subsequently boosted after challenge becoming at least three-fold higher than that those against Peptide-1. Both control ponies developed antibody responses against the two antigens after challenge which, in contrast to the pre-immunised ponies, were at similar levels for both antigens.

To study further the antigenic differences exhibited in the VN test by the deletion mutant and wild type viruses, a panel of sera collected from experimentally infected ponies was analysed in a cross neutralisation test against CVL Bucyrus and EAV-G_LΔ viruses (Table 3.8). These ponies were infected with one of the following viruses: LP3A, LP3A⁺, UK93 (a second passage in BHK-21 of a semen isolate collected from the UK outbreak in 1993), pf (a pleural fluid isolate of velogenic Bucyrus), sp (a small plaque variant of pf obtained by three cycles of plaque picking) and 'vero-adapted' (a derivative of pf obtained after 30

passages in Vero cells). The results show that, with the exception of samples from the vero-adapted and EAV-G_LΔ infected ponies, all the antisera neutralised efficiently the wild type CVL Bucyrus presenting VNAb titres above 2. Conversely, only EAV-G_LΔ post-infection sera neutralised at a high titre the EAV-G_LΔ virus. Interestingly, the VNAb titres against the deletion mutant virus of vero-adapted sera were low too. These data altogether supports the hypothesis that EAV-G_LΔ differs antigenically from wild type EAV strains. This might be the case as well for the vero-adapted EAV strain.

3.4. Discussion

In the work presented above, the potential of a genetically modified EAV virus presenting a deletion in the immunodominant region of the G_L ectodomain as a marker vaccine has been explored. As a live marker vaccine this virus should present the following features:

a) expression *in vivo* of those proteins relevant for the induction of immune response; b) safety for the individual, therefore non-pathogenic, and safety for the environment; c) induction of an immune response allowing serological distinction from natural infection and d) induction of protective immunity against infection.

In this pilot study we have performed for the first time an infection of ponies with EAVG_LΔ to evaluate how well this potential marker vaccine complies with the above mentioned requirements. In particular, the study was designed to test whether the virus retained its infectivity and determine the outcome of the infection in terms of pathogenicity and immunogenicity.

The administration route and virus dose used are known to infect ponies reliably using other EAV strains and both ponies inoculated with EAVG_LΔ became infected. The experiment showed, consistent with experiments performed *in vitro* in RK-13 cells and EDC, that despite deletion of parts of G_L, the virus conserved its infectivity *in vivo* since virus was isolated repeatedly from nasal swabs and blood from the ponies. This is consistent with a recent study showing that chimeric EAV viruses in which the G_L ectodomain was replaced by that of PRRSV or LDHV were still infectious to BHK-21 and RK-13 cells (Dobbe *et al.*, 2001).

Despite being infected with EAVG_LΔ virus, the animals remained asymptomatic during the infection and the levels of virus excretion were low, suggesting the possible attenuation of this virus. However, the present experiment did not permit the assignment of this apparent attenuation to the genetic manipulation introduced since virus derived from the full length G_L plasmid pEAN515 was not tested in parallel with EAVG_LΔ virus. Comparison with the results of an experimental infection of horses with the virus derived from the full length clone pEAV030 (Balasuriya *et al.*, 1999b) does not provide a conclusive answer since duration of viraemia and nasal excretion were similar to those described for EAVG_LΔ virus infected ponies but, unlike our study, both ponies became febrile albeit mildly. Furthermore, EAV often occurs asymptotically in the field and experimental infections with some isolates (McCollum and Timoney, 1998; Castillo-Olivares, unpublished observations) produced no remarkable clinical signs. Both ponies infected with EAVG_LΔ presented viraemia and therefore there is a potential for causing abortion in the pregnant mare. Also, the fact that EAV-

G_LΔ can be excreted from the upper respiratory tract, although at lower titres than wild type EAV strains, and is infectious by the natural route could be undesirable features of this virus as a vaccine. Continuous transmission of EAV in horses could result in the generation of novel genetic variants with increased pathogenicity. The generation of new genetic variants has been described in persistently infected stallions and this has been identified as the origin of an outbreak (Balasuriya *et al.*, 1999a). Also the attenuated live PRRSV vaccine has been demonstrated to be transmitted from vaccinated pigs to susceptible animals with the appearance of novel genetic variants which are more pathogenic (Nielsen *et al.*, 2001). Further studies are required to evaluate attenuation in more detail. In particular, with regards to the abortigenic potential of the recombinant virus, reversion to virulence and capacity to establish persistent infections in stallions.

The present study allowed us to evaluate the immunity against EAV infection provided by exposure to the deletion mutant virus. Both ponies inoculated with EAV-G_LΔ mounted an immune response to EAV as evidenced by the detection of antibodies in serum to whole G_L ectodomain and by the demonstration of VNAb to wild-type EAV. Complete clinical protection against the virulent LP3A⁺ virus was observed accompanied by only occasional isolation of challenge virus from nasal swabs and blood in the vaccinated ponies. This level of protection was unexpected in the light of the relatively low VNAb titres (1.2 and 0.8) against LP3A⁺ at the time of challenge. These VNAb's were stimulated after EAV-G_LΔ infection and boosted after challenge despite the G_L ectodomain being partially deleted from the immunising virus. Consequently, these

antibodies must either recognise epitopes in the modified G_L polypeptide (antigenic sites A and B as identified by Balasuriya *et al.* (1997) are still present in the deletion mutant virus) or be directed to - yet unidentified - neutralising epitopes lying outside the G_L protein. However, it seems unlikely that VNAb alone can have provided the levels of protection observed. Previous studies (Chapter 2) showed that ponies vaccinated with a recombinant protein comprising the entire G_L ectodomain presenting titres around 1.0 at the time of challenge were only partially protected, despite these ponies showing a strong anamnestic response after infection. More likely, the intranasal administration of a replicative immunogen may have stimulated a wide range of immune effector mechanisms, not dependent upon the deleted region of the G_L protein. Thus, strong VNAb responses directed to epitopes outside the G_L(66-112) region may have been elicited efficiently, particularly at the natural port of virus entry in the nasal mucosa. Alternatively, cell-mediated immunity may have contributed to the protection against challenge infection. Antibody-dependent cell-mediated cytotoxicity (ADCC) and cytotoxic T lymphocytes have been described in the horse (Allen *et al.*, 1994; O'Neill *et al.*, 1999; McGuire *et al.*, 1997). Recently, methods for the detection of EAV specific cytotoxic responses have been developed and the induction of CD8⁺ CTL responses following wild-type EAV infection has been demonstrated (Chapter 4). These and other methods should enable us to characterise the immune effector mechanisms that are stimulated after immunisation with EAV-G_LΔ and to evaluate the relative importance of antibody- versus cell-mediated mechanisms.

The serology results indicate that the deletion mutant virus stimulates an antibody response that would allow differential diagnosis with natural infection

making it a potential marker vaccine. Following EAV-G_LΔ immunisation, antibody responses were detectable but were different to those following wild type infection. Thus, VNAb was preferentially directed against the deletion mutant virus rather than wild type EAV, and anti-Peptide 1 responses could not be detected by ELISA. In contrast, all post-challenge sera reacted to the Peptide-1 antigen and neutralised very efficiently wild type EAV viruses. The different antigenic properties shown by the serology results of the four ponies used in this study were confirmed by analysis of the virus neutralisation capacity of a panel of post-infection antisera. The poor neutralisation of wild type EAV by EAV-G_LΔ antisera and conversely poor neutralisation of EAV-G_LΔ by wild type EAV antisera suggests that there are no other VN epitopes apart from those in G_L and that the deletion in this protein has resulted in the formation of a new antigenic site targetted by the immune system that results in virus neutralisation *in vitro*.

The use of the full-length clone has proved to be a very valuable tool to construct a potentially efficacious marker vaccine and can be used to dissect the effector mechanisms of the adaptive immune response and understanding their role in protection.

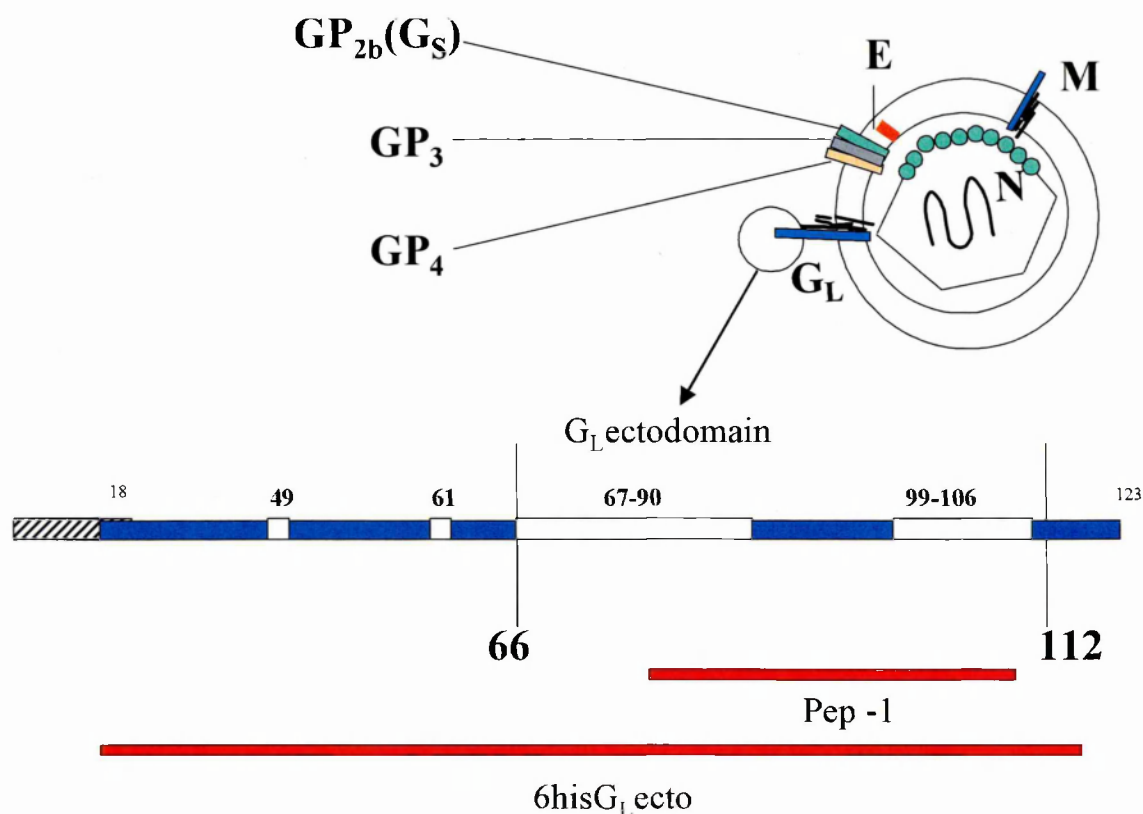


Figure 3.1. Schematic representation of the EAV virion. The regions of the G_L ectodomain that contribute to form neutralising epitopes are indicated by the white boxes. The size of deletion in the G_L ectodomain which was made to generate EAV-G_LΔ is indicated by the two vertical lines. The two G_L derived antigens used to analyse the antibody responses of ponies to EAV-G_LΔ and EAVLP3A⁺ inoculation is indicated by the red lines. The putative signal sequence is indicated by the hatched box.

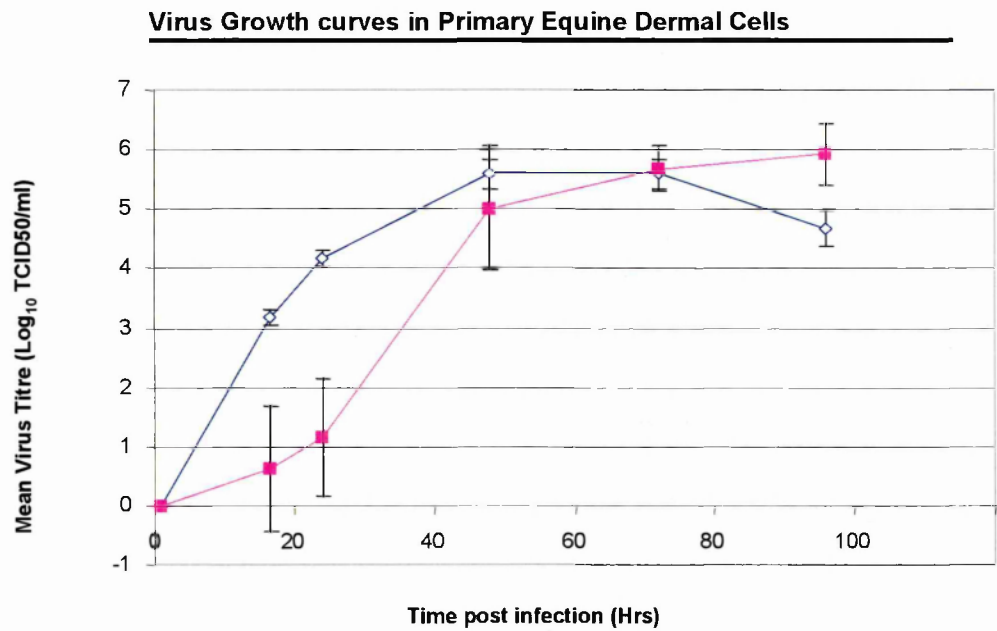


Figure 3.2. Low multiplicity of infection (m.o.i. = 0.001) virus growth curves of LP3A+ (blue line) and EAV-GLΔ (red line) viruses

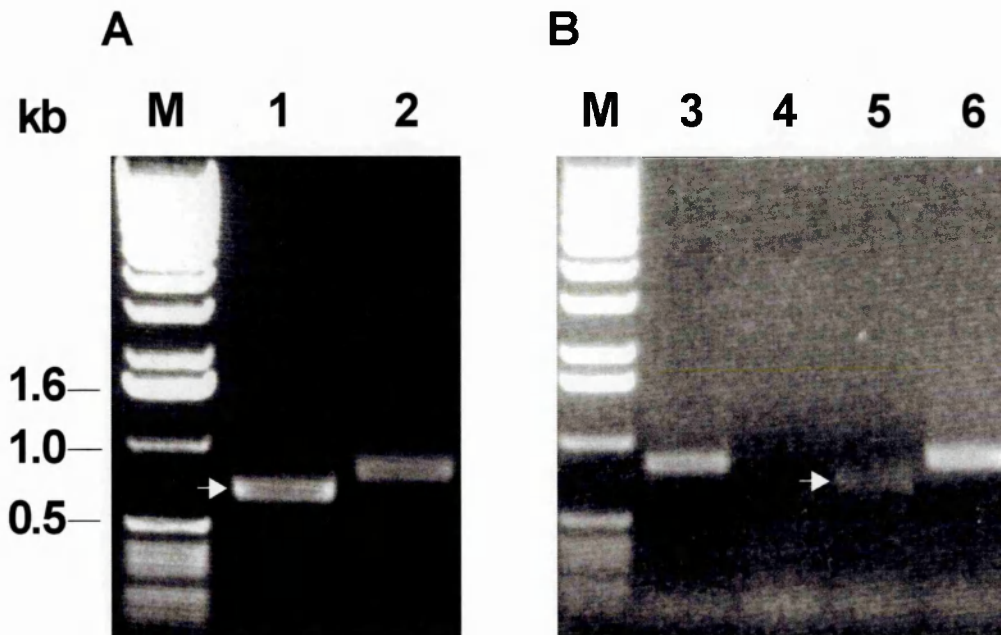
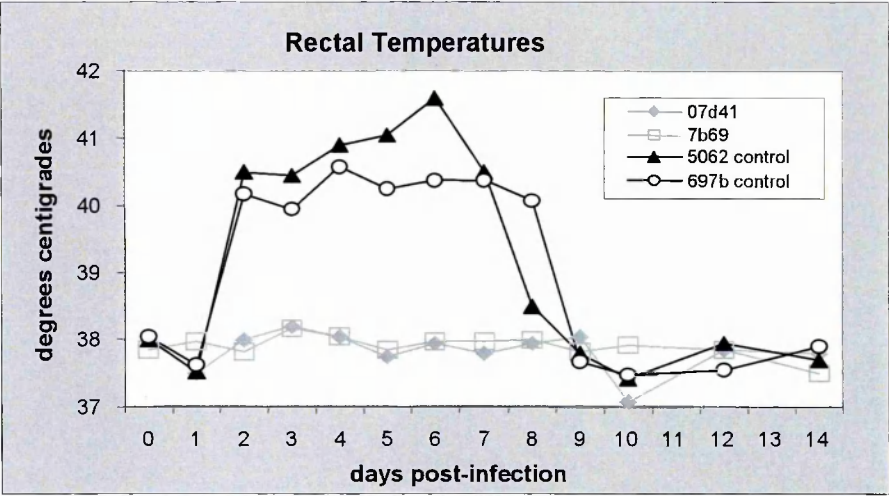


Figure 3.3. RT-PCR analysis of ORF5 from EAV-G_LΔ inoculated into and retrieved from ponies. RT-PCR amplification of ORF5 was carried out on tissue culture fluid, using primers JV1 and JV2. The presence of the deletion in ORF5 was checked both of the input virus and of virus present in the blood of one of the ponies (#7b69) at day 4 after inoculation with EAV-G_LΔ. Lane 1: EAV-G_LΔ inoculum virus prepared in EEL cells; lanes 2 and 3: wild type EAV strain CVL Bucyrus grown in RK13 cells; lane 4: uninfected RK13 tissue culture fluid; lane 5: virus recovered in RK13 cells from EAV-G_LΔ infected pony (#7b69); lane 6: challenge virus (LP3A+) prepared in EEL cells. On the left the positions and sizes of marker DNA fragments are indicated (in nucleotides).

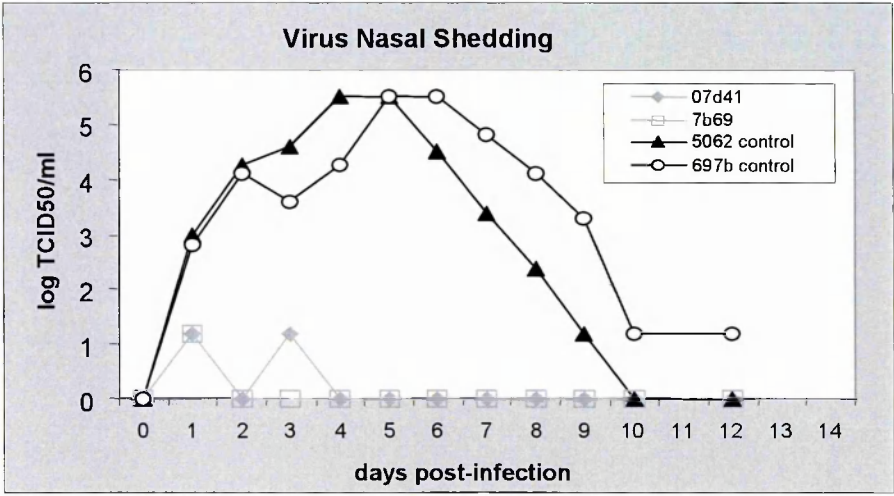
days	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
virus isolation from swab		+	+	+		+	+	+			neg		neg		neg	Pony 07d41
virus isolation from hep			neg		neg		+		+		+		+		+	
Rectal Temperature	37.8	37.9	37.4	37.8	37.7	38.1	37.8	37.9	38		38		37.8			
virus isolation from swab			+	+	+	+	+	+			neg		neg		neg	Pony 7b69
virus isolation from hep			neg		+		+		+		neg		+		+	
Rectal Temperature	37.6	37.9	37.4	37.8	37.9	38.1	37.95	38.1	37.7		38		38			

Table 3.4. Pyrexia, virus nasal excretion and viraemia from ponies 7b69 and 07d41 over a 14 day period following intranasal infection with EAVG_LΔ .

a)



b)

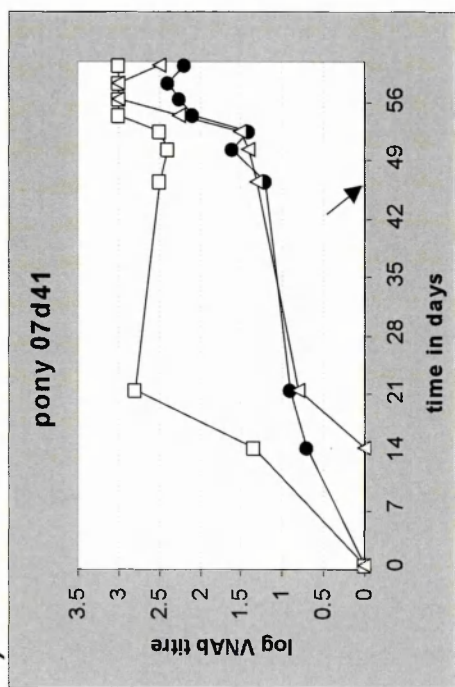


c)

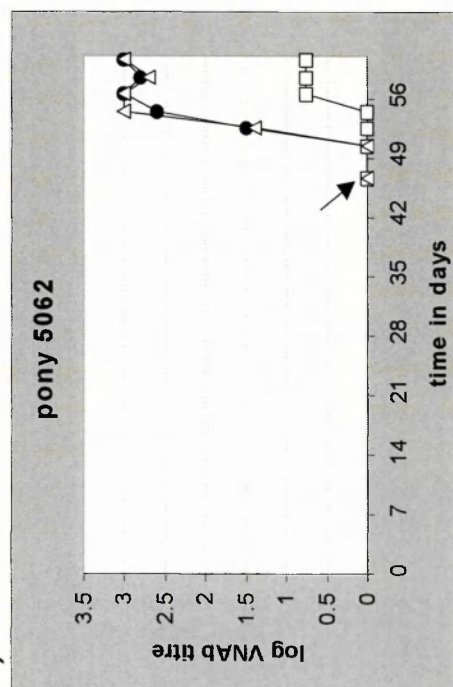
Pony	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	19	21
07d41			neg		neg		neg		+		neg		neg		neg		
7b69			neg		neg		neg		+		neg		neg		neg		
5062			+		+		+		+		+		+		+	+	+
697b			+		+		+		+		+		+		+	+	+

Figure 3.5. Rectal temperature (a), nasal virus excretion (b) and cell associated viraemia (c) of pre-vaccinated (7b69 and 07d41) and control ponies (5062 and 697b) following intranasopharyngeal challenge with EAV LP3A+ strain. Shaded boxes indicate samples not taken.

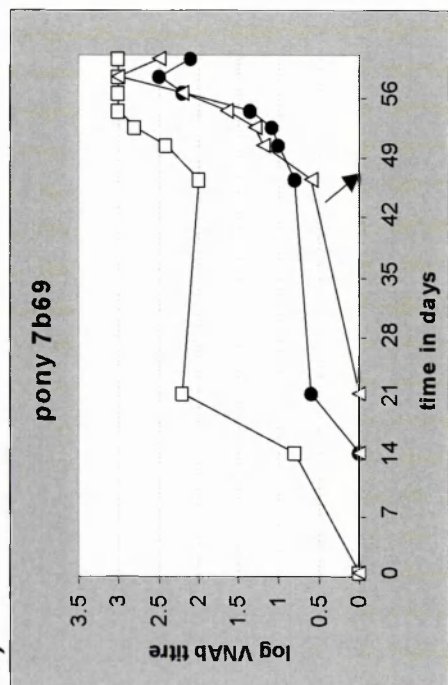
a)



c)



b)



d)

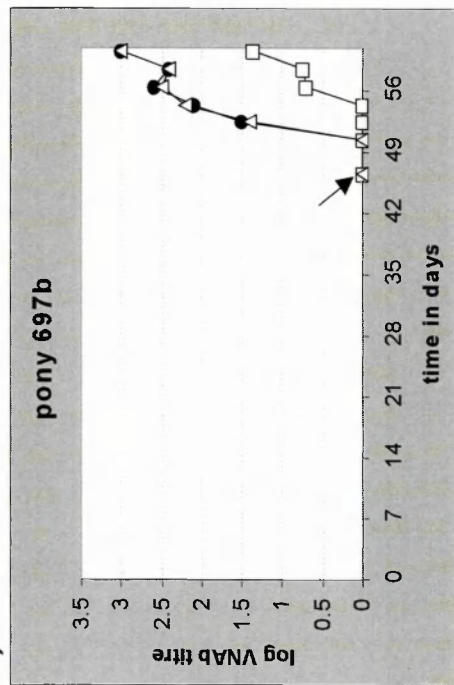
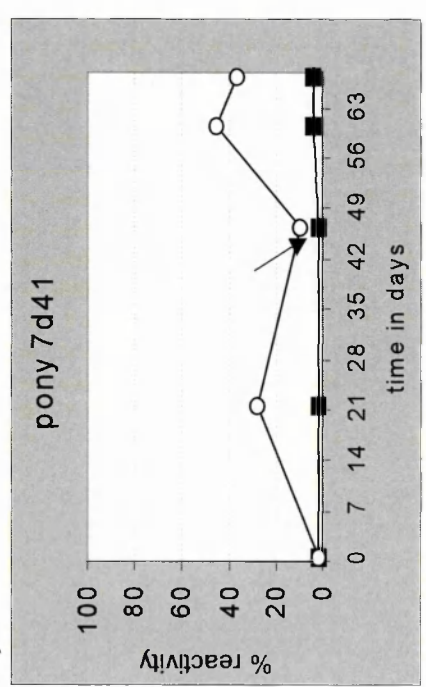


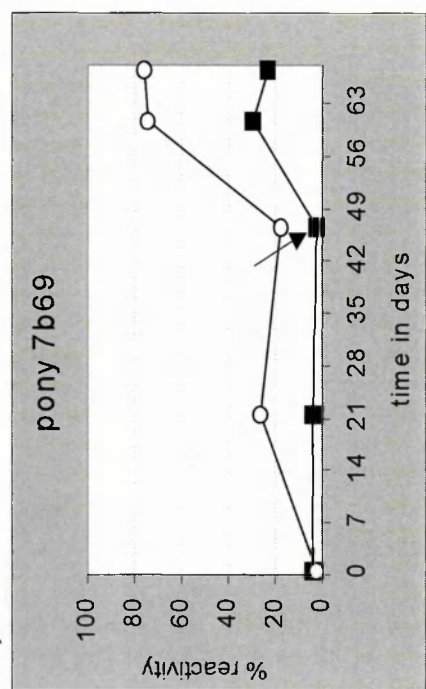
Figure 3.6. Virus neutralising antibody responses in serum of ponies 7b69, 07d41, 5062 and 697b against EAV-G_LΔ (-□-), LP3A⁺ (-●-) and Bucyrus CVL (-Δ-) after intranasopharyngeal vaccination (ponies 7b69 and 07d41) with EAV-G_LΔ (day 0) and intranasopharyngeal challenge infection with EAV LP3A⁺ (day 46; arrow).

■ Pep-1
 ○ 6hisG_L

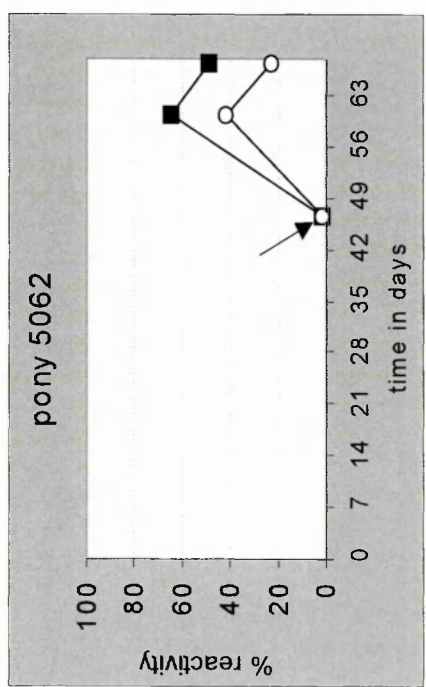
a)



b)



c)



d)

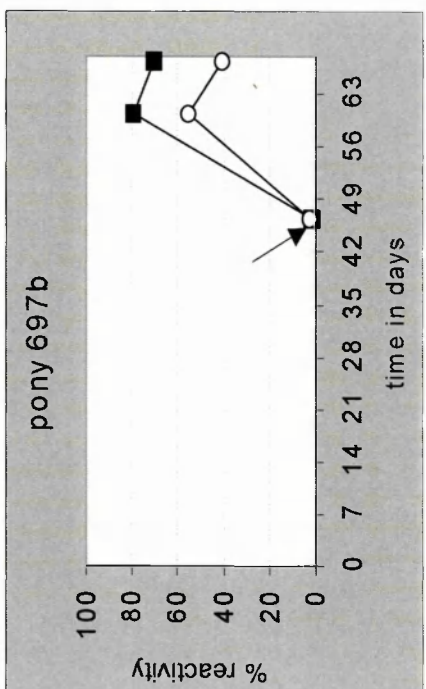


Figure 3.7. G_L protein specific antibody responses of ponies 7b69, 07d41, 5062 and 697b after intranasopharyngeal immunization (ponies 7b69 and 07d41) with EAV-G_LΔ (day 0) and intranasopharyngeal challenge infection with EAV LP3A+ (day 46; arrow) measured by ELISA against antigens 6HisG_L(G_L residues 18-122) and Peptide-1 (G_L residues 81-106). Reactivities of sera are expressed with respect to a positive control serum sample obtained from pony p6, 2 weeks post-vaccination with 6HisG_L antigen (14).

Pony ID	Challenge virus	Time of sampling	VN Ab titres	
			CVLBucyrus	GLdeletion mutant
7b69	GLdeletion mutant	21	<0.6	2.2
07d41	GLdeletion mutant	21	0.6	1.9
6d2c	LP3a	28	2.4	0.75
3566	LP3a	28	2.3	0.75
1757	pf	29	2.9	0.9
2d3e	pf	29	2.5	0.7
035e	vero adapted	28	0.6	1.05
3227	vero adapted	28	0.75	0.8
6540	sp	7 month	2.55	1.05
3d1a	sp	7 month	2.5	1.05
297e	LP3a	14	1.95	<0.6
chnt	LP3a	14	2.25	1.05
37c	uk93	40	2.5	<0.6
gp	uk93	33	2.8	0.75
bp	uk93	33	2.8	<0.6
7378	LP3a+	30	2.85	0.75
5d66	LP3a+	30	3.4	1.4
027a	LP3a+	30	2.85	1.05

Table 3.8. VNAb titres (expressed in log10) against CVL Bucyrus and EAV-G_LΔ of a panel of sera collected from ponies experimentally infected with different EAV strains.

Chapter 4: Detection of EAV-specific Cytotoxic T Lymphocyte Responses from EAV Infected Ponies.

4.1 Introduction

Virus neutralising antibodies (VNAb) are believed to play an important role in the immunity against equine viral arteritis (Fukunaga *et al.*, 1981; McCollum, 1969). Their appearance in serum coincides with clinical recovery and reduction of virus excretion, passive transfer of colostrum antibodies from immune mares to foals was found to moderate or prevent equine viral arteritis (McCollum, 1976) and protection in animals immunised with inactivated (Fukunaga *et al.*, 1984, 1990) or G_L-sub-unit vaccines (Castillo-Olivares *et al.*, 2001, Chapter 2) correlated with VNAb titres at the time of challenge. Currently, nothing is known about cell mediated immunity (CMI) to EAV such as the role it plays in clearance of virus infection and whether it would be desirable to stimulate such immunity by vaccination. However, virus specific cytotoxic lymphocytes have been detected in mice infected with the arterivirus LDV (Even *et al.*, 1995, van den Broek *et al.*, 1997) and there are some indications that CMI plays an important role in clearance of SHFV infections since persistently infected patas monkeys with low titre VNAb's can clear subsequent acute infections caused by a different strain (Gravell *et al.*, 1986a). Furthermore, EAV-G_LΔ immunised ponies with low VNAb titres against LP3A+, were highly protected against this strain, suggesting the involvement of immune effector mechanisms in addition to VNAb's in protection from EAV (Chapter 3).

Animals that recover from EAV infection develop a long lasting immunity against the disease (Gerber *et al.*, 1978) although not always against reinfection (McCollum, 1969). However, EAV replication in chronically infected stallions, which is restricted to cells of the accessory sex glands, persists for several months or years despite high levels of circulating VNAb's. EAV is able to establish persistent infections in the reproductive tract of the stallion and the cell associated viraemia that occurs soon after infection lasts from one to several weeks after serum VNAb's become detectable (Neu *et al.*, 1987; Castillo-Olivares, Unpublished observations). The role that immune effector mechanisms other than VNAb's play in viral clearance during acute or chronic infections is largely unknown due to the lack of methods to study these responses.

The aim of the following work is to determine whether EAV induces cytotoxic CMI responses and to examine some of their features in the natural host. To do this work, cell targets (targets) were collected from experimentally infected ponies and the activity of EAV-specific cytotoxic cells (effectors) was measured using a standard ^{51}Cr release assay. In order to study cytotoxic T lymphocyte (CTL) responses to EAV, the following ponies were used:

- ponies 7378, 027a and 5d66 (infected with LP3A+ to validate this virus stock as a suitable challenge virus for vaccine efficacy studies)
- ponies 697b, 5062, 7b69 and 07d41 (used to evaluate the immunity provided by the EAV-G Δ virus - chapter 3).

Skin biopsies were taken from the ponies before infection with EAV to prepare primary cell lines to be used as targets in CTL assays and blood samples were collected at various times after infection to isolate PBMC's for use as 'effectors'.

4.2. Materials and Methods

4.2.1. *Animals*

Seven two-year-old castrated male ponies were used to obtain peripheral blood mononuclear cells (PBMC) and equine dermal cells (EDC). Both types of cells were used in assays to detect EAV-specific CTL responses. Ponies 7378, 027a, 5d66, 5062 and 697b were infected intranasally with $10^{6.1}$ TCID₅₀ of the EAV LP3A⁺ strain and ponies 07d41 and 7b69 were immunised with EAVGL Δ and subsequently challenged with EAVLP3A⁺. Clinical signs and antibody responses of ponies 7b69, 07d41, 697b and 5062 to EAVGL Δ and / or EAVLP3A⁺ infections have been described previously (Chapter 3). EAVLP3A⁺ infections of ponies 7378, 5d66 and 027a resulted in a moderate-severe acute EAV syndrome characterised by pyrexia, anorexia, lethargy, weight loss, mild ataxia and conjunctivitis, similar to those described for ponies 5062 and 697b. Virus was isolated from nasal secretions for the first week of infection and from blood up to day 21 post infection. VNAb were first detected in serum by day 6 post infection, rose to titres of 1/100 and remained stable for more than a year.

4.2.2. *Viruses and cells*

A derivative of the EAV strain LP3A (Chapter 2) was used for in vitro infections and for the experimental infection of ponies. This virus, designated EAV LP3A⁺, was obtained by a single passage of EAV LP3A in equine embryonic lung cells (EEL) using a low multiplicity of infection. The Diagnostic Virology Laboratory of the Animal Health Trust supplied this primary cell line, obtained from an aborted foetus. The cell line was tested to be free of mycoplasma or equine

viruses. The cells were maintained in MEM [minimum essential medium Eagle with Earle's salts (Sigma, M2279), 100 IU of penicillin per ml, 10 µg of streptomycin per ml, non-essential amino acids, L-glutamine to a final concentration of 2 mM] and supplemented with 10% heat inactivated fetal bovine serum (10% MEM). Equine dermal cells (EDC) obtained from the ponies used in this study via skin punch biopsies were maintained in 10% MEM or 20% MEM.

4.2.3. Radiolabelling of EDC

EDC monolayers, prepared as described in section 4.3.1., were washed twice with PBS and then trypsinised. Once detached, the cells were resuspended in 10% MEM and centrifuged for 5 min. at 475 g. and resuspended in 20% MEM at 4×10^5 cells / ml. The cells were inoculated with EAV LP3A+ at a m.o.i. of 0.3 TCID₅₀ / cell or left uninfected. Then, Na⁵¹CrO₄ (Amersham) was added to each cell suspension to a final concentration of 1-1.5 µCi / 4×10^4 cells before distributing volumes of 100µl into 96 well flat bottomed plates. The plates were incubated at 37°C, 5% CO₂ for 24 hours.

4.2.4. Secondary stimulation of in vivo primed PBMC (effectors)

The induction of cytotoxic cells from PBMC was conducted essentially as described for detection of EHV-1 specific CTL by Allen *et al.* (1995) with minor modifications. Briefly, whole venous blood was collected at various times after infection into vacuum tubes containing 1 IU of sodium heparin in PBS / ml of whole blood, and then the mononuclear cell fraction isolated by Ficoll-Hypaque

density gradient centrifugation. PBMC were collected from the interface, washed 3 times in PBS to reduce the number of platelets and resuspended in either freezing medium (10% dimethylsulfoxide, fetal bovine serum) or induction medium [1:1 vol/vol mixture of AIM-V (Gibco) / RPMI 1640 (Sigma) supplemented with 2mM L-glutamine, minimal essential medium non-essential amino acids (0.05 mM each), 0.5 mM sodium pyruvate, 2-mercaptoethanol (55 μ M), gentamicin (50 μ g/ml) and equine serum (7% - collected from the ponies before EAV experimental infection and inactivated at 56°C for 40 min)]. The PBMC were incubated in induction medium for 7 days in upright 75 cm² tissue culture flasks at $1.1 - 2.0 \times 10^8$ cells / flask / 40 mls in the presence or absence (for preparation of mock-induced effector cells) of $10^{6.1}$ TCID₅₀ of EAV LP3A+.

4.2.5. Measurement of cytolytic activity of 'in vitro' stimulated PBMC.

EAV-induced and mock-induced PBMC cultures were centrifuged at 800 g, 20°C, 10 min, without brake, the supernatant was discarded and cell pellets resuspended in CTL medium (RPMI 1640 containing 10% heat inactivated equine serum). Both cultures were adjusted to contain the same concentrations of viable cells (determined by trypan blue exclusion) and diluted appropriately in CTL medium to obtain different effector: target ratios when added to the overnight grown fibroblasts. Target cells (24 hr after addition of ⁵¹Cr, +/- EAV infection) were washed 3 times with RPMI 1640 using 125 μ l / well in each wash before the addition of either the effectors, a CTL medium control or cell lysis solution (2% Triton-X100 in PBS). The plates were incubated for 4 hours at 37°C, 5% CO₂ after which the supernatants were harvested (Supernatant

Harvesting System, Skatron, Newmarket, Suffolk) for quantitation of ^{51}Cr release by gamma counting. The lytic activity of each PBMC culture dilution was assessed against 4 – 6 replicates of EAV infected and uninfected autologous or allogeneic radiolabelled targets. The percentage of specific ^{51}Cr release was calculated according to the formula: $[(e - sp) / (t - sp)] \times 100$, where e is the experimental ^{51}Cr release in the presence of effectors, sp is the spontaneous ^{51}Cr release in the presence of CTL medium, and t is the total ^{51}Cr release from targets incubated with cell lysis solution.

4.2.6. Indirect immunofluorescence

Equine dermal fibroblasts or cytopins of PBMC were fixed in acetone or 4% formaldehyde, 0.4% Triton-X 100 in PBS for 15 min at room temperature. After washing in PBS, an anti-EAV nucleocapsid (N) specific rabbit polyclonal antiserum (de Vries *et al.*, 1992) diluted 1/100 in PBSA (2% bovine serum albumin, PBS) was applied to the cells and incubated for 1 hour at 37°C. After washing in PBS, the samples were incubated for 1 hour at 37°C with an anti-rabbit IgG FITC conjugated antibody (DAKO, Denmark) diluted 1/40 in PBSA, washed again in PBS and observed using a fluorescence microscope.

4.2.7. Flow cytometry

Equine dermal cells were pre-treated before immunostaining procedures in suspension were carried out. Confluent monolayers of EDC were trypsinised, washed in 20% MEM, resuspended in 20% MEM and incubated at 37°C, 5% CO_2 for 2 hours. *In vitro* induced effectors and pre-treated targets were washed in

PBSG (0.5% normal goat serum, 0.01% sodium azide, PBS) 3 times and 2×10^6 cells resuspended in PBSG containing monoclonal antibodies (MAb) (2 μg / ml) specific for equine lymphocyte antigens (Lunn *et al.*, 1998). Cells were incubated with MAb H58A, H42A, HT14A or HB61-A (VMRD Inc., Pullman, USA) specific for MHC-I, MHC-II, CD8 and CD4 antigens respectively. Background staining was determined using an IgG1 isotype control MAb (DAKO, Denmark). After 1 hour incubation on ice, the cells were washed with ice cold PBSG and resuspended in goat anti-mouse IgG FITC conjugated antibody (DAKO, Denmark) diluted 1/40 in PBSG and incubated for 1 hour in ice. After a final wash in PBSG the cells were fixed in 2% formaldehyde and analysed on a FACScalibur flow cytometer (Beckton Dickinson). Dead cells were identified using propidium iodide staining at a concentration of 20 μg / ml.

4.2.8. Separation of CD4⁺ and CD8⁺ T cells from induced PBMC cultures

Biomagnetic separation of effector cells was performed using the MACS Goat anti-mouse IgG microbeads and MS Separation Columns (Miltenyi Biotec, Germany) following essentially the manufacturer's recommendations. Briefly, after 7 days incubation, the PBMC cultures were centrifuged, the supernatant discarded and the cells washed twice in separation buffer (PBS, 0.5% BSA, 2mM EDTA). The stimulated PBMC were then incubated for 1 hour at 4°C in separation buffer with or without 2 μg /ml of either anti-equine CD4 MAb HB61A or anti-equine CD8 MAb HT14A. The cells were washed twice in separation buffer and incubated with the anti-mouse IgG conjugated microbeads for 1 hour at 4°C before being washed again in separation buffer and applied to the separation columns. Both enriched and depleted cell fractions were collected,

centrifuged and the cell pellets resuspended in CTL medium to obtain equivalent concentrations of effectors. The cells were then assayed for cytotoxic activity as described above.

4.3. Results

4.3.1. Preparation of cytotoxic cell targets.

4.3.1.1. Establishing Primary Equine Dermal Cell (EDC) lines from skin biopsies.

Primary EDC lines were established for each pony used in the study. Skin punch biopsies were performed under aseptic conditions, the epidermal portion of the sample removed and the dermal plug immersed in 20% MEM and transported to the laboratory for immediate processing. The dermal plug was sliced with the help of sterile scalpel blade and forceps and each slice laid on a well of a 6 well flat bottomed plate. Fresh 20% MEM was added to each well and the plates incubated at 37°C, 5% CO₂ for five days. Once islets of fibroblasts were observed growing, the inoculum was removed and fresh 20% MEM was added and incubation continued at 37°C, 5% CO₂ until a confluent monolayer was formed. Then the cells were passaged to a 25 cm² tissue culture flask, expanded and finally resuspended in 10% DMSO in foetal calf serum (FCS) for cryopreservation in liquid nitrogen. All the experiments were performed with cells between passages 5 and 14.

4.3.1.2. Infection of Equine Dermal Cells with EAV.

EDC's can support EAV replication, but in order to use them in a cytotoxicity assay, a high proportion should be viable and express viral antigen at the time

they are incubated with the effectors. Virus titration of EAV in EDC's and other cell lines indicated that at certain virus dilutions the cell sheets did not show cytopathic effect until 48 hours post-inoculation, retaining their normal morphology for the first 24 hours. To check the proportion of cells expressing viral antigens at this time post-infection, EDC suspensions in 10% MEM infected with LP3A+ at different multiplicities of infection were seeded on a flat bottomed 96 well plate at 4×10^4 TCID₅₀ / well. After a 24 hour incubation period, the cells were washed and fixed with formaldehyde-Triton-X-100 (4% formaldehyde, 0.4 % Triton-X-100, PBS) and EAV N antigen expression checked by immunofluorescence. The results indicated that at a m.o.i. of 0.3, antigen expression was observed in a high proportion of cells and the cells conserved their normal morphology (Fig 4.1).

4.3.1.3. Expression of MHC-I and MHC-II on the surface of EDC's

Cytotoxic T lymphocytes recognise non-self antigens through interaction of their T cell receptor and co-receptor (CD4 or CD8) with processed peptides displayed by MHC-I or MHC-II molecules on the surface of cells. With a few exceptions, such as neurons, MHC-I molecules are present in all nucleated cells, although the levels of expression vary between different tissues. In contrast, expression of MHC-II is restricted to endothelial cells and various cells of the immune system. To check whether EDC's express MHC-I or MHC-II after being processed in the laboratory, cell suspensions were subjected to immunofluorescence staining with specific MAb's and flow cytometry scanning. The results (Figure 4.2) showed that EDC's express MHC-I but not MHC-II, indicating they could be used as

targets recognised by CD8⁺ CTLs, but not by CD4⁺ lymphocytes restricted by MHC-II.

4.3.1.4. Retention of ⁵¹Cr

Standard ⁵¹Cr release cytotoxicity assays are based in the property of viable cells taking up Cr in the form of Na₂⁵¹CrO₄, which is then released very slowly to the extracellular medium. When cell damage occurs, the isotope complexes are released and the γ -radiation emitted by the extracellular medium can be measured as an indication of cell lysis. In order to test whether EDC's could absorb and retain ⁵¹Cr, two sets of EDC suspensions in 20% MEM containing 4x10⁵ cells/ml were labelled with 10 μ Ci/ml of Na₂⁵¹CrO₄. One was infected with EAV LP3A+ at a m.o.i. of 0.3. The other was left un-infected. Both sets of cells were dispensed in 12 wells of a flat bottomed 96 well plate and incubated for 24 hours at 37°C, 5% O₂. After incubation, the plates were washed 3 times with 150 μ l / well of serum-free MEM and the cells from each set were incubated for 4 hours with 100 μ l / well of either 2% Triton-X-100 solution or MEM containing 10% of inactivated normal autologous equine serum (CTL medium). The liquid contents of each individual well were harvested and radioactivity measured. The results (Table 4.3) show little variation between wells containing the same type of cells and a percentage spontaneous ⁵¹Cr release of 18.5% and 15.3% for the EAV infected and un-infected cells respectively.

These studies showed that EDC's expressed MHC-I molecules and that 24 hours EAV infection at an m.o.i. of 0.3 the majority of cells expressed EAV antigen and retained normal morphology. Moreover, these cells were successfully

labelled with ^{51}Cr and showed sufficiently low spontaneous release of isotope to function as viable targets for CTL.

4.3.4. Induction of cytotoxic cells (effectors) from PBMC of EAV convalescent ponies.

Peripheral blood mononuclear cells obtained from EAV infected ponies were used as the source of EAV-specific cytotoxic cells. PBMC's were obtained by standard methods and were stored in liquid nitrogen or used directly for stimulation with EAV. Stimulation of CD8⁺ cytotoxic cells against EHV-1 has been achieved by incubation of PBMC's with virus for 6 days without the addition of external cytokines (Allen *et al.*, 1995). This procedure was used to induce EAV-specific cytotoxic cells.

First attempts to detect EAV specific cytotoxicity were performed using cryopreserved PBMC's. After thawing, the PBMC's were centrifuged to remove the DMSO and were resuspended in Induction Medium. The PBMC's were split into 2 sets, one of which was inoculated with LP3A⁺ virus. EAV-induced and mock-induced PBMC cultures were processed as indicated in 4.2.5. and incubated with EAV-infected and uninfected targets. Radioactivity of each individual well was measured and the percentage specific lysis calculated.

A cytotoxicity assay, performed with cryopreserved effectors derived from pony 5d66 PBMC collected 21 days post-infection and autologous targets (Table 4.4), showed very low levels of specific lysis for the 4 combinations of effectors and targets used. In a second assay, cryopreserved PBMC's collected 42 days post-infection from pony 7378 were induced and cytotoxicity measured against EAV-

infected and uninfected autologous targets. In this assay the effector: target ratios were increased with respect to the previous assay and N antigen expression of EAV infected EDC's was checked by immunofluorescence at the time effectors were added to the targets. Again, there was no evidence for EAV-specific cytotoxicity (Table 4.5) even though immunofluorescence results (not shown) indicated that approximately 80% of the cells were expressing EAV N protein and the viability of the effectors was approximately 80% as determined by Trypan Blue exclusion staining. The assay was repeated again using cryopreserved PBMC's but produced similar results (not shown).

Monocytes and macrophages are the main cell targets of arteriviruses. Damage of these cells during freezing and thawing processes of PBMC could have resulted in the depletion of EAV permissive cells in the induction cultures, thereby preventing virus antigen expression, protein processing and presentation of virus peptides by MHC-I on the surface of antigen presenting cells. Consequently, a new cytolysis assay was set up using fresh PBMC's in the induction cultures. This time, effectors derived from pony 7378 PBMC's collected 4 months post-infection showed EAV specific cytolytic activity. The specific lysis induced by EAV-stimulated effectors on EAV-infected targets was more than double that for mock-infected targets or induced by mock-stimulated PBMC's (Fig. 4.6).

Hematoxylin-eosin staining of 7 day-old PBMC cultures showed a marked reduction in the numbers of monocytes in the EAV induced effectors compared with the mock-induced effectors (Fig 4.7). Samples from the EAV induced PBMC's taken 24 hours after virus inoculation revealed EAV N antigen expression on the PBMC's (Figure 4.7c). The morphology of the infected cells

suggested that they were monocytes. To check whether cryopreservation could have damaged EAV-permissive PBMC subpopulations in the induction cultures, cryopreserved PBMC's isolated from pony 5062 before infection and collected fresh 6 weeks post-infection (viraemia was cleared by week 4 post-infection) were infected with EAV using the same conditions as those described above. Twenty-four hours after inoculation with the virus, the cells were resuspended, washed, fixed and checked for N antigen expression by immunofluorescence. Only freshly collected PBMC's were positive (data not shown).

4.3.5. Effects of equine serum origin on the induction of cytotoxic cells.

Pre-infection autologous serum was used to prepare the induction medium used in the above experiments and also in the first 2 assays performed to evaluate genetic restriction of EAV-specific cytotoxicity (section 4.3.6). Since the availability of this reagent posed a limitation to the number of assays that could be performed, an experiment was set up to test whether the use of heterologous equine serum affected the generation of cytotoxic cells. A pool of 4 EAV VN negative sera, obtained before infection from ponies 5062, 697b, 07d41 and 7b69, was used as the heterologous serum. Two sets of induction medium whose composition differed only in the source of equine serum (autologous or heterologous) were used to prepare EAV stimulated and mock-induced effectors. Cytotoxic effector cells were prepared from PBMC collected from pony 027a 6 months post-infection. The lytic activity of the effectors was tested against EAV infected or uninfected targets. The results (Figure 4.8) show no difference between the specific lysis obtained by either set of EAV-induced effectors

demonstrating the adequacy of heterologous serum as a component of induction medium.

4.3.6. Genetic restriction of EAV specific cytotoxicity.

The ponies used as sources of effector cells in these studies were from an outbred population and it was not possible to determine their MHC-I haplotype. To determine whether the virus-specific cytolytic activity exhibited by EAV stimulated effectors collected from convalescent ponies was genetically restricted, a series of cross-matching cytotoxicity assays were performed. In these experiments, EAV induced and mock-induced PBMC's from one individual were incubated with autologous or allogeneic EAV or mock-infected targets. The first of the experiments (Fig 4.9.a) showed EAV induced 7378 PBMC's collected 6 months post-infection specifically lysed autologous and 027A derived EAV-infected targets. All uninfected and 5D66 derived EAV infected cells showed low levels of lysis. Assuming that cytolysis was mediated by MHC-I restricted CTL, the data suggested that pony 7378 shared a MHC-I allele with pony 027A but not with 5D66. To test this hypothesis, a second cross matching cytolysis experiment was carried out (Figure 4.9.c) in which PBMC's collected 6½ months post-challenge from pony 5D66, were incubated with EAV-infected or uninfected EDC's from ponies 5D66, 7378 and 027A. Only killing of autologous EAV infected targets was observed, supporting the hypothesis that cytolysis was genetically restricted by MHC-I. In a further experiment, EAV-induced and mock-induced PBMC from pony 027A, collected 8 months post-infection, were incubated with infected and uninfected targets from the three ponies. This time, 027A effectors recognised EAV-infected target cells from

ponies 027A and 7378, but not uninfected cells or EAV-infected cells from pony 5D66 (Fig 4.9.e). Mock-induced effectors did not show significant specific cytolytic activity in any of these experiments (Figs 4.9.b, 4.9.d and 4.9.f).

4.3.7. Attempts to block effector cytotoxicity with anti-MHC-I monoclonal antibody.

The results of the CTL assays presented above suggest that EAV-specific cytotoxicity was mediated by MHC-I restricted CD8⁺ T lymphocytes. Blocking of MHC-I molecules on the targets with specific MAb's was performed to obtain further evidence for the MHC-I restricted nature of cytotoxicity. In this experiment, effectors (EAV- and mock-induced) derived from the PBMC of pony 5d66 collected 7 months post-infection were split into 2 sets. Set A effectors were used in a standard CTL assay. Set B cells were used in a CTL assay at an effector: target ratio of 50:1 in which the targets contained 3µg, 1µg or 0.3µg per well of anti-MHC-I MAb H58A. The results (Fig. 4.10) showed that the presence of the anti-MHC-I antibody did not prevent the EAV specific cytotoxicity.

4.3.8. Further analyses of genetic restricted cytotoxicity

The genetic restriction of the EAV specific cytotoxicity was examined further using effectors and targets derived from another group of ponies that underwent an EAV experimental infection. A cytotoxicity assay using effectors derived from pony 7d41 obtained 5 months after challenge and targets originated from ponies 697b, 5062, 7b69 and 7d41 showed again the genetic restricted nature of the EAV specific cytotoxicity exhibited by EAV induced effectors (Fig. 4.11). Effectors derived from pony 7d41 recognised only autologous targets.

A second cytolysis assay was performed with this set of 4 targets using effector cells induced from PBMC's collected 6 months post-infection from ponies 697b and 7b69. The results of this experiment (Fig. 4.12) showed an EAV-specific cytotoxic response of 697b effectors, which was restricted to autologous targets. In contrast, 7b69 effectors showed a more promiscuous killing causing specific lysis of EAV infected targets from ponies 7d41, 7b69, 697b and 5062 (Fig. 4.12). It should be noted that relatively high levels of lysis were obtained in all targets after incubation with mock-induced 7b69 effectors in contrast to those obtained with mock induced 697b effectors. The relatively high lysis levels produced by 7b69 effectors in this assay (both on infected and uninfected targets) opens the possibility of the activation of phenotypically different subpopulations of cytotoxic effector cells, including non-MHC restricted killer cells. Lymphokine activated killer cell (LAK) mediated cytotoxicity has been demonstrated in the horse as well as in other species. These cells can lyse, following *in vitro* stimulation with IL-2, targets from an equine lymphoma cell line (T8888) but not xenogeneic targets such as Daudi and EL4 cells and suggested these cells are of the T-cell lineage (Hormanski *et al.*, 1992). Viveiros *et al.* (1999) characterised further the cytolytic activity of equine NK and LAK cells showing that K-562 cells (MHC-I negative) could be lysed by both LAK and NK cells whereas T8888 cells (MHC-I positive) could only be lysed by LAK cells. These effectors could have proliferated and been stimulated in this particular assay.

The promiscuous killing of effectors derived from pony 7b69 was not so evident in a further experiment designed to test the effects in the assay of AIM-V medium as a component of the Induction Medium. PBMC's from pony 7b69

collected 7 months post-infection were used to prepare 2 sets of induction cultures using either complete Induction Medium or Induction Medium deprived of AIM-V medium. Each set was split into 2 subsets, one inoculated with EAV and the other left uninfected. After a 7-day incubation period at 37°C in a 5% CO₂ atmosphere, the PBMC's were used in a cytotoxicity assay. The results (Fig. 4.13) showed a higher cytolytic activity of the effectors derived from PBMC's incubated in induction medium containing AIM-V. Both sets of effectors however produced similar results showing EAV-specific cytotoxicity against targets from 7b69 and 697b but not from 7d41. In this case however, mock stimulated effectors showed less cytolytic activity than in the previous experiment. The cytotoxicity observed in this assay was virus induced, exerted over infected cells only and appeared to be genetically restricted suggesting it was T-lymphocyte mediated. The lysis of only autologous infected targets by 697b effectors and the lysis of autologous and 697b infected targets by 7b69 effectors could be the result of pony 697b sharing an MHC-I allele with 7b69, being this MHC-I superseded by other alleles in the presentation of EAV antigens to CD8 T-lymphocytes during the natural infection.

In summary, the results of the cross-matching assays suggest, but do not confirm, that the virus specific cytotoxicity observed in our cytotoxicity assays is mediated by CD8⁺ MHC-I restricted CTL. Testing the cytolytic activity of specific subpopulations of lymphocytes from the effectors' pool was carried out to further characterise the EAV specific cytotoxic responses.

4.3.9. Phenotype of cytotoxic effector cells

In order to determine whether the cytotoxic effectors were CD8⁺ or CD4⁺ cells, biomagnetic selection and depletion of specific lymphocyte subpopulations of effectors and subsequent analysis of their cytolytic properties was conducted. EAV-induced and mock-induced effectors derived from pony 697b PBMC collected 7 months post-infection were split into 2 subsets. One of them was processed directly for measurement of EAV-specific cytolytic activity. The other subset was processed for selection and depletion of CD4⁺ or CD8⁺ T cell populations as indicated in materials and methods, and assayed for cytolytic activity. The concentrations of the selected and depleted effectors were intended to be adjusted to obtain effector: target ratios of 6:1. However, in this particular experiment it was not possible to obtain this ratio with the CD8⁺ selected effectors and these were used at a 1:1 ratio. The results displayed in Fig. 4.14 show, as expected, very little cytolytic activity of all subpopulations derived from mock-induced effectors. Unselected and CD4^{-ve} and CD8^{-ve} effectors from EAV induction cultures lysed EAV-infected targets in contrast to CD4⁺ve selected T cells. It is difficult to compare the levels of killing of CD8⁺ve selected cells since they were used at an effector: target ratio of 1: 1 only. These results indicated that not all effectors were CD8⁺ T cells or that the selection procedure of CD8⁺ T cells was sub-optimal.

A second experiment was conducted using EAV-induced effectors only. This time the MAb's were used at a concentration of 2 µg / 10⁷ cells and a control of the selection procedure was included in that PBMC's were subjected to

biomagnetic selection in the absence of CD4 or CD8 specific MAb's. The unprocessed effectors were used at a 25:1 ratio with respect to the targets whereas the selected effectors were used at a 6:1 ratio. Immunoperoxidase staining of acetone-fixed PBMC collected from the depleted fractions with monoclonal antibodies to CD4 and CD8 antigens showed a complete absence of CD8⁺ cells and almost complete absence of CD4⁺ cells in the CD8 and CD4 depleted populations respectively. In contrast, both CD4⁺ and CD8⁺ T-lymphocytes were present in the buffer control depleted sample. The results of the cytolytic assay (Fig. 4.15) show that only the CD8⁺ enriched fraction of the PBMC and the untreated effectors induced high percentages of cytolysis of EAV infected targets. In contrast, the CD8⁺ depleted and the CD4⁺ enriched cell subpopulations presented very low cytolytic activities. These results suggested that the *in vitro* EAV-specific cytotoxicity observed in all these experiments was mediated by CD8⁺ T-lymphocytes. The presence of these cytotoxic lymphocytes could explain the moderate levels of EAV-specific cytolysis induced by the CD4⁺ depleted effectors and the negative cell fraction of the buffer separation (Buffer control negative).

Further characterization of the effectors was performed by flow cytometry. Following 7 days culture, EAV-induced and mock-induced cultures from 5062 PBMC collected one year after infection were analysed for CD4 and CD8 expression. Light scatter analysis of both sets of effectors show two cell subpopulations in contrast to freshly isolated PBMC. In a previous experiment, propidium iodide staining of EAV- and mock-induced effectors showed that one of these subpopulations contained many dead cells (Fig. 4.16) and hence only

live cells were gated (R1) for phenotypic analysis of the effector cell population. Incubation of PBMC in the presence of EAV resulted in a lower percentage of live cells in comparison with the mock-induced cultures. However, EAV-induced effectors showed a higher proportion of cells with increased size and granularity (region R2), indicative of activated lymphoblasts. In addition, EAV-induced PBMC displayed an upregulation of either CD4 or CD8 on a sub-population of cells, as evidenced by a second peak of higher fluorescence intensity for CD4⁺ and CD8⁺ cells. Overall, there was an increase in the CD8/CD4 ratio (from 0.43 to 0.77) comparing the mock- with EAV-induced cultures, resulting from an increase in the proportion of CD8⁺ cells and decrease in the proportion of CD4⁺ cells in the R1 gate. Comparison of the number of cells within the R2 gate compared with the R1 gate demonstrated an increase in the proportion of both CD4⁺ and CD8⁺ cells within the activated lymphoblast population, comparing mock-induced versus EAV-induced cultures. Thus, the proportion of CD4⁺ cells in the R2 gate increased from 9% to 30% and of CD8⁺ cells from 18% to 42% (relative to the total number of viable CD4⁺ or CD8⁺ cells). Furthermore, the majority of CD4⁺ and CD8⁺ cells present within the R2 gate for the EAV-induced cultures displayed upregulated expression of CD4 or CD8 respectively. These data indicate activation of both CD8⁺ and CD4⁺ lymphocytes in response to EAV induction.

4.3.10. EAV specific cell-mediated cytotoxicity before and at early stages of convalescence.

To obtain further evidence for the nature of the EAV-specific CTL responses, cytotoxicity assays were performed using effectors derived from PBMC collected

from pony 5062 before infection, one month post-infection and 3 months post-infection (Fig. 4.18). Mock-induced effectors showed low levels of specific lysis for all targets whether they were infected or not. As expected, EAV-induced effectors prepared from PBMC collected from the pony before EAV infection did not produce significant lysis of EAV-infected autologous targets or of targets derived from pony 7378. However, it was unexpected to observe relatively high specific lysis of EAV-infected targets derived from pony 697b. Unfortunately, allogeneic mock-infected targets were not included in this assay. One month post-infection, EAV-induced cytotoxicity was observed for EAV-infected autologous targets as well as both infected and mock-infected 027a derived EDC. Cytotoxicity of 697b targets was not observed in this assay. The results obtained 3 months post-infection showed elevated levels of lysis only in autologous EAV infected targets, being consistent with previous results.

4.4. Discussion

Understanding virus-host interactions during equine viral arteritis has been hampered by the lack of methods to measure cellular immune responses to this virus. The work presented describes for the first time methods to assess cytotoxic T-lymphocyte responses in ponies against EAV. The methods employed were essentially those described by Allen *et al.* (1995) and O'Neill *et al.* (1999) with some modifications. The first of these was the use of primary EDC's as virus-infected target cells instead of *in vitro* stimulated lymphoblasts. A few attempts to detect EAV N antigen in lymphoblasts after virus inoculation proved unsuccessful. The use of dermal cells as targets for cytotoxic assays has been documented in immunological studies performed in species other than equine

(Onishi *et al.*, 1999; Sharpe *et al.*, 2001; Korsoy *et al.*, 2001; Flomemberg *et al.*, 1996). In other studies in horses, detection of cytotoxic cellular immunity has been achieved by using methods employing mitogen stimulated lymphoblasts (Hannant and Mumford 1988; Allen *et al.*, 1996; O'Neill *et al.*, 1999), primary kidney fibroblasts (McGuire *et al.*, 1997) and primary equine dermal cells (Hannant, personal observations) as targets. The present study has found EDC easy and convenient to use. These cells are more easily accessible than equine kidney cells and do not require previous incubation under special conditions (in contrast to mitogen stimulated lymphoblasts). Moreover, they retain chromium efficiently and support EAV replication. However, they have the disadvantage of not constitutively expressing MHC-II which excludes the possibility of measuring CD4⁺ mediated cytotoxicity and so the significance of this mechanism *in vivo* remains unknown.

In the present study, generation of virus-specific CD8⁺ T lymphocytes was achieved by incubating the PBMC for a few days in the presence of live virus without adding exogenous cytokines such as IL-2. Previously, it has been shown that there is a positive correlation between the amount of exogenous IL-2 in induction cultures and the promiscuity of equine cytotoxic effector cells for influenza virus infected targets (Hannant and Mumford 1988). It is worth noting however, that unlike EHV-1 CTL induction, cytotoxic activity against EAV infected cells could not be induced using cryopreserved mononuclear cells. This is probably due to the damage of EAV permissive subpopulations of cells during the freezing and / or thawing processes. In this regard, it should be noted that T-lymphocytes, the major population of PBMCs sensitive to EHV-1 infection, are

not affected by the freezing and thawing process. In EAV-specific cytolytic assays, the number of monocytes in EAV-induced cultures (7 days post-induction) much reduced in comparison to the mock-induced PBMC. Moreover, EAV N antigen expression was clearly detected in 24 hours-old EAV-induced cultures prepared from fresh but not cryorecovered PBMC. Taken together, these observations suggest that EAV-infected monocytes (principal cell target of arteriviruses *in vivo*) act as the main antigen presenting cells to CD8+ T cells and CD4+ T cells through MHC-I and MHC-II molecules respectively. Activation of CD4+ T cells would result in production of IL-2, which in turn would help expansion and differentiation of CD8+ T cells into cytotoxic effectors. Monocytes are cleared from the cultures by day 7 post-induction as a result of virus infection or by cell-mediated cytotoxicity. Alternatively, antigen presentation to CD4+ T cells could have been provided by virus specific B cells.

EAV-specific cytotoxic cells have been induced from PBMC collected from EAV convalescent ponies over a period of 1 year post-infection. These cells, generated after *in vitro* stimulation with virus antigen, expressed CD8+ and showed a genetically restricted cytolytic activity, possibly associated with recognition of virus derived peptides presented by MHC-I molecules, although this could not be confirmed directly by blocking of MHC-I with MAb H58A. However, this reagent has not been used before to block CTL lysis and it is possible that binding of the MAb to its epitope does not interfere with peptide antigen recognition on the target cell. Determination of the equine leucocyte antigen of the ponies used in the study (which was not possible at the time these experiments were performed), would have helped to definitively corroborate the

MHC-I restriction of the cytolytic activity. However, there is strong evidence for EAV-specific cytotoxic effector cells recognising EAV-infected target cells via MHC-I presentation because a) only MHC-I antigen expression (and not MHC-II) could be detected on EDC, b) the cytotoxic memory response was genetically restricted, c) the reduction of monocytes from the EAV induced effectors, making unlikely that the cytotoxicity was mediated by macrophages recognising infected cells via Fc receptor IgG interactions and d) cytotoxic cells were CD8⁺ T-lymphocytes.

Clinical recovery from EAV and the reduction in virus excretion from nasal secretions coincides with the development of VNAb in serum (usually after the first week of infection), as observed in the ponies used in this study and described in other reports (Fukunaga *et al.*, 1981; McCollum *et al.*, 1969; Castillo-Olivares *et al.*, 2001, Chapter 2). However, cell associated viraemia persists for longer periods ranging from 2 or 3 weeks after infection to around 3 months post-infection (Neu *et al.*, 1987; Castillo-Olivares, unpublished observations). Whether the resolution of viraemia is the result of a cell-mediated clearance mechanism or due to the eventual disappearance of leucocytes infected during the first few days is not clear but is consistent with cytotoxic lymphocytes eventually eliminating virus infected cells in blood and other tissues. In this study, the EAV infected ponies recovered well from the infection and were the source of PBMC's used as effectors in the cytotoxicity assays.

The detection of CD8⁺ T lymphocyte precursors from EAV convalescent ponies indicates that cellular immunity might play an important role in the final

clearance of the infection. The temporal development of CTL responses was studied in one pony but the results from sequential cytotoxicity assays performed with 5062 effectors showed that the capacity of the effectors to recognise and lyse infected targets increased during the first 3 months post-infection, point at which the immune system probably returns to a basal resting state characterised by the predominance of memory cells in the circulating PBMC. By 1-3 months, cell-associated viraemia in the majority of LP3A+ infected ponies was cleared. In PRRSV infections in pigs it appears that a combination of cellular and humoral immune clearance mechanisms are necessary to clear infection. This infection induces an antibody response to viral antigens which is detectable by day 9 post-infection (Labarque *et al.*, 2000) but lymphoproliferative responses and neutralizing antibodies were not detectable until week 4 post-infection (Lopez Fuertes *et al.*, 1999) coinciding with clearance of viral infection from blood and lungs. Observations in mice infected with LDV indicate that cytotoxic lymphocytes are stimulated but disappear 30 days later leading to a life-lasting persistent infection (Even *et al.*, 1995). This study supports the hypothesis that CTL's contribute to the resolution of EAV viraemia in ponies. However, the mechanism of viral persistence in LDV infections, which involves a process of clonal deletion of new LDV-specific CD8+ cells in the thymus leading to a state of tolerance to LDV-CTL epitopes, is probably different to that occurring in EAV persistently-infected stallions. Further analysis of additional immunological parameters is required to define precisely the mechanisms of viral clearance or persistence in EAV infections.

The detection of CD8⁺ T cytotoxic precursors cells during equine viral arteritis and their probable contribution to EAV clearance, together with the high levels of immunity provided by live vaccines (Doll et al., 1968; McCollum, 1970; McCollum *et al.*, 1986) and the deletion mutant EAV-G_LΔ (Chapter 3), indicate that marker vaccination approaches would benefit from the induction of this type of immune response. Incorporation of virus proteins containing CTL epitopes common to most equine MHC-I haplotypes as well as choosing an appropriate antigen delivery system capable of inducing the processing of proteins by the endocytic pathway for antigen presentation by MHC-I would be necessary for the design of marker sub-unit vaccines stimulating CTL responses.

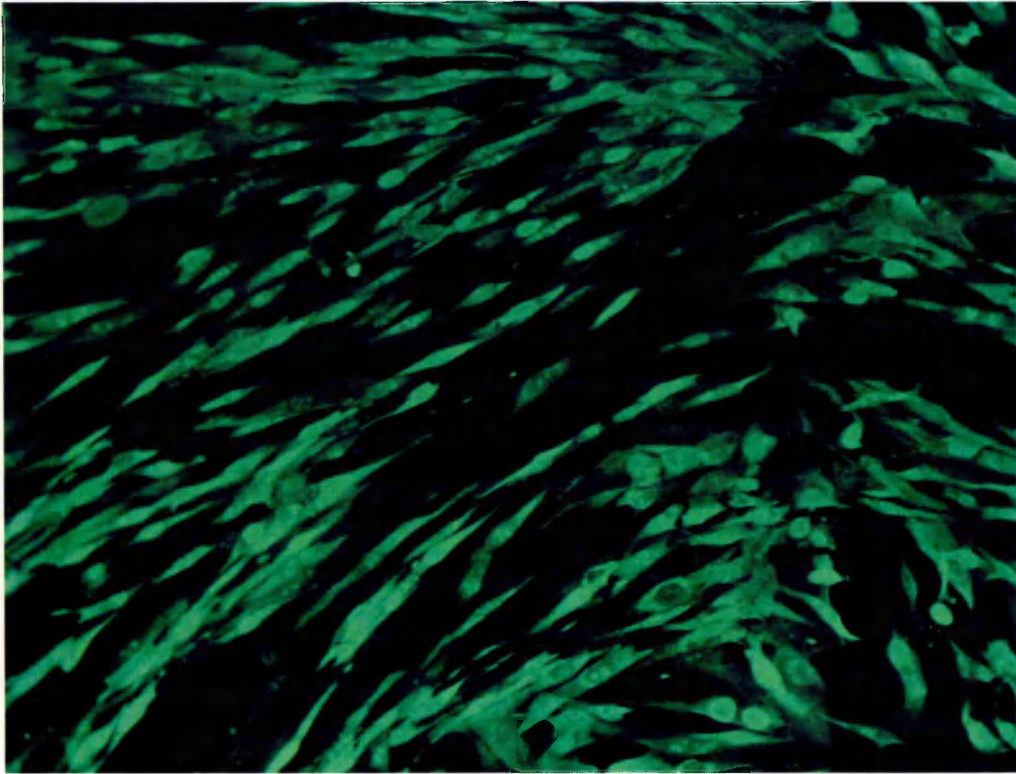


Figure 4.1. EAV nucleocapsid protein (N) expression in EDC 24 hours after infection with EAV at an m.o.i. of 0.3. Detection was performed by indirect immunofluorescence using SP07 rabbit antibody and FITC conjugated goat anti-rabbit IgG.

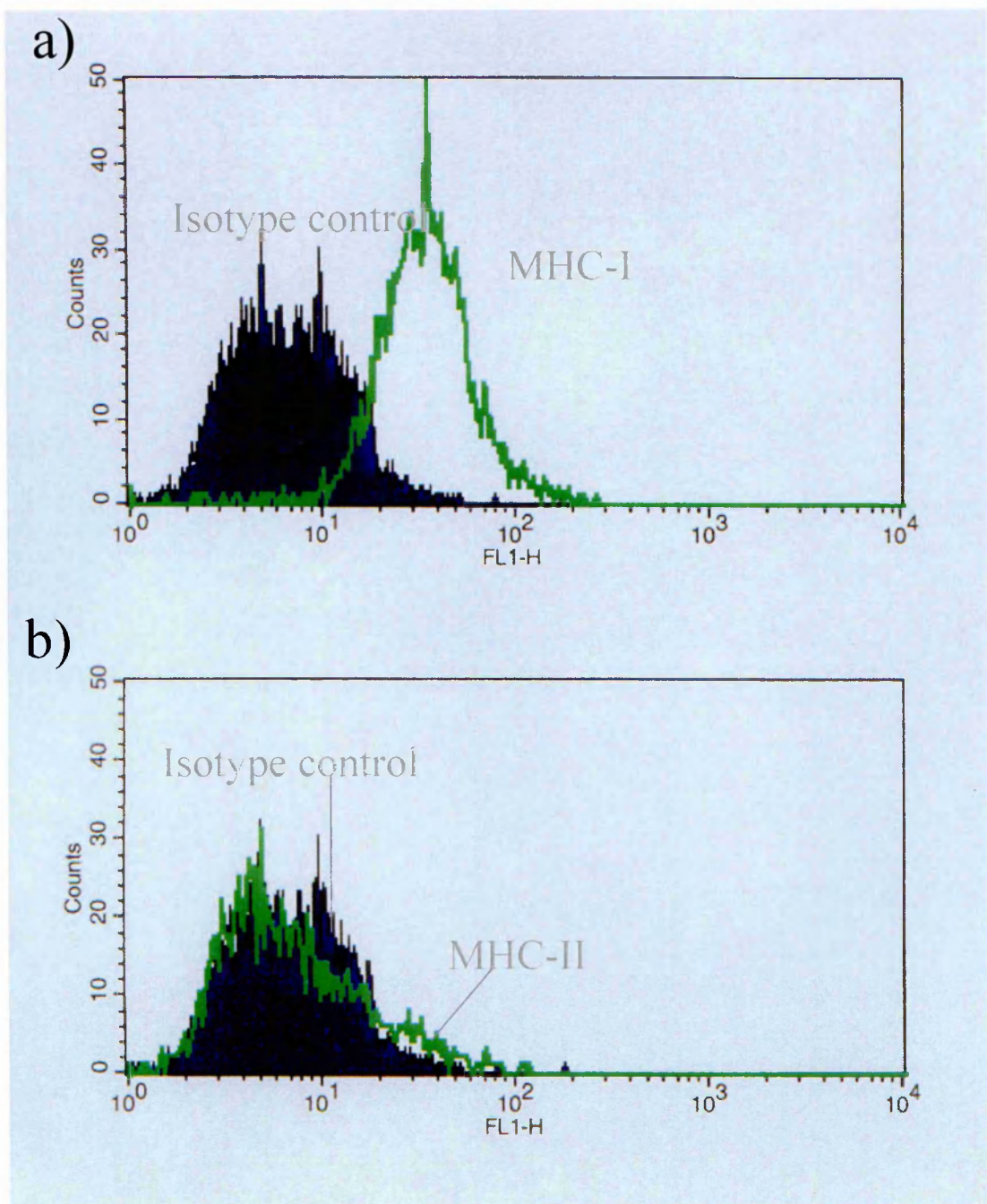


Figure 4.2. Expression of MHC molecules on EDC.

Suspensions of EDC were incubated with MAb specific for either equine MHC-I (H58A), equine MHC-II (H42A) or equine CD8 (HT14A - serving as an isotype control). MAb binding was detected using FITC-conjugated goat anti-mouse IgG. Panels a) and b) show cell-surface expression of MHC-I and MHC-II respectively (open histograms); in both panels the isotype control is shown as a closed histogram

Medium		EAV infected EDC's			Non-infected EDC's		
		Mean cpm	STDV	%STDV	Mean cpm	STDV	%STDV
Spontaneous release	CTL medium	3309.2	104.1	3.1	2840.9	145.8	5.1
Total release	2%TX100	17930.3	508.0	2.8	18543.0	225.7	1.2
% Spontaneous release		18.5			15.3		

Table 4.3. Capacity of EDC’s to retain and release ⁵¹Cr under the physical conditions of the CTL assay. The mean and standard deviations of 6 replicate samples are shown

Effectors	E:T ratio	EAV infected targets		Mock infected targets	
		mean cpm	% specific lysis	mean cpm	% specific lysis
EAV induced	10:1	2240.67	1.10	2302.67	2.58
	5:1	2036.50	-1.87	1941.33	-2.24
	2.5:1	2007.50	-2.29	1960.83	-1.98
Mock induced	10:1	2033.67	-1.91	2071.83	-0.50
	5:1	2040.33	-1.82	2004.33	-1.40
	2.5:1	1986.17	-2.61	2002.17	-1.43
TritonX100	tot.Cr release	9029.17	100.00	9604.83	100.01
10% RPMI	sp. Cr release	2165.67	23.00	2109.33	22.00

Table 4.4. Cytotoxic activity exhibited against autologous targets by in vitro stimulated effectors derived from cryopreserved PBMC collected from pony 5d66 on day 21 post-infection.

Effectors	E:T ratio	EAV infected targets		Mock infected targets	
		cpm	% specific lysis	cpm	% specific lysis
Eav induced	50:1	2864.43	1.77	2450.52	0.56
	25:1	2791.64	1.00	2291.56	-0.77
	12.5:1	2735.63	0.41	2520.91	1.15
Mock induced	50:1	2763.38	0.70	2456.02	0.61
	25:1	2777.83	0.86	2545.77	1.36
	12.5:1	2809.31	1.19	2512.80	1.08
2%TritonX100	tot Cr release	12213.71	100.00	14314.50	100.00
10%RPMI	sp Cr release	2696.32	22.08	2383.66	16.65

Table 4.5. Cytotoxic activity exhibited against autologous targets by in vitro stimulated effectors derived from cryopreserved PBMC collected from pony 7378 on day 42 post-infection.

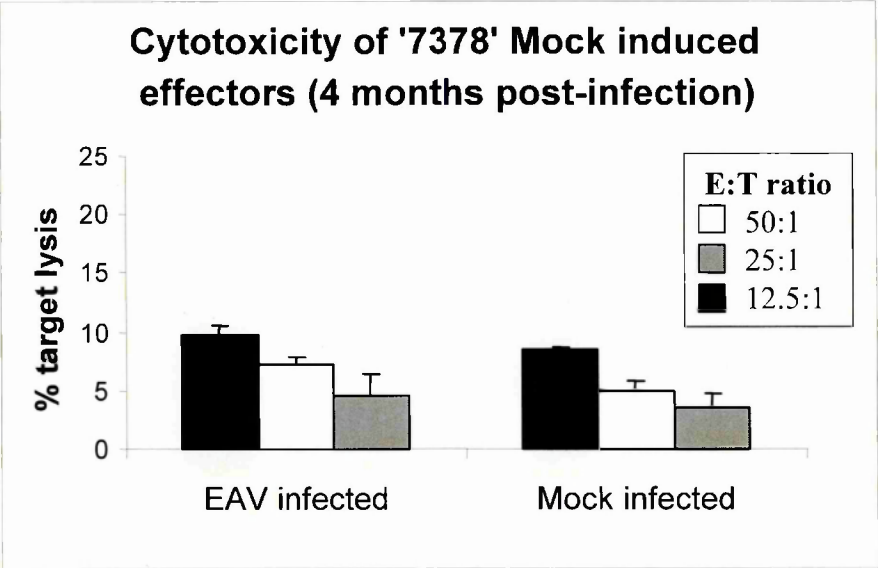
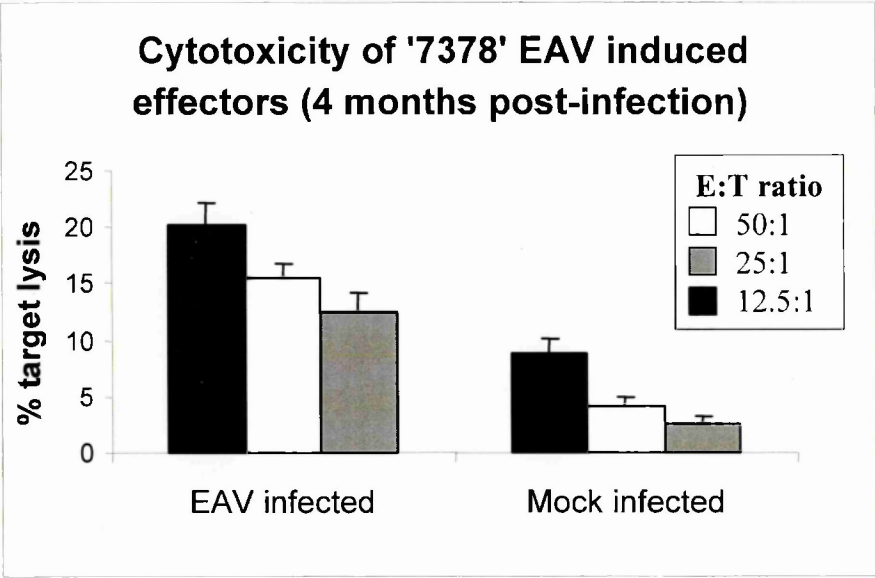


Figure 4.6. Cytotoxic activity displayed against autologous targets by in vitro stimulated PBMC collected from pony 7378 four months post-EAV infection. The PBMC were used fresh for in vitro induction. Results show the % specific lysis of targets at different effector: target cell ratios; 50:1, 25:1 and 12.5:1.

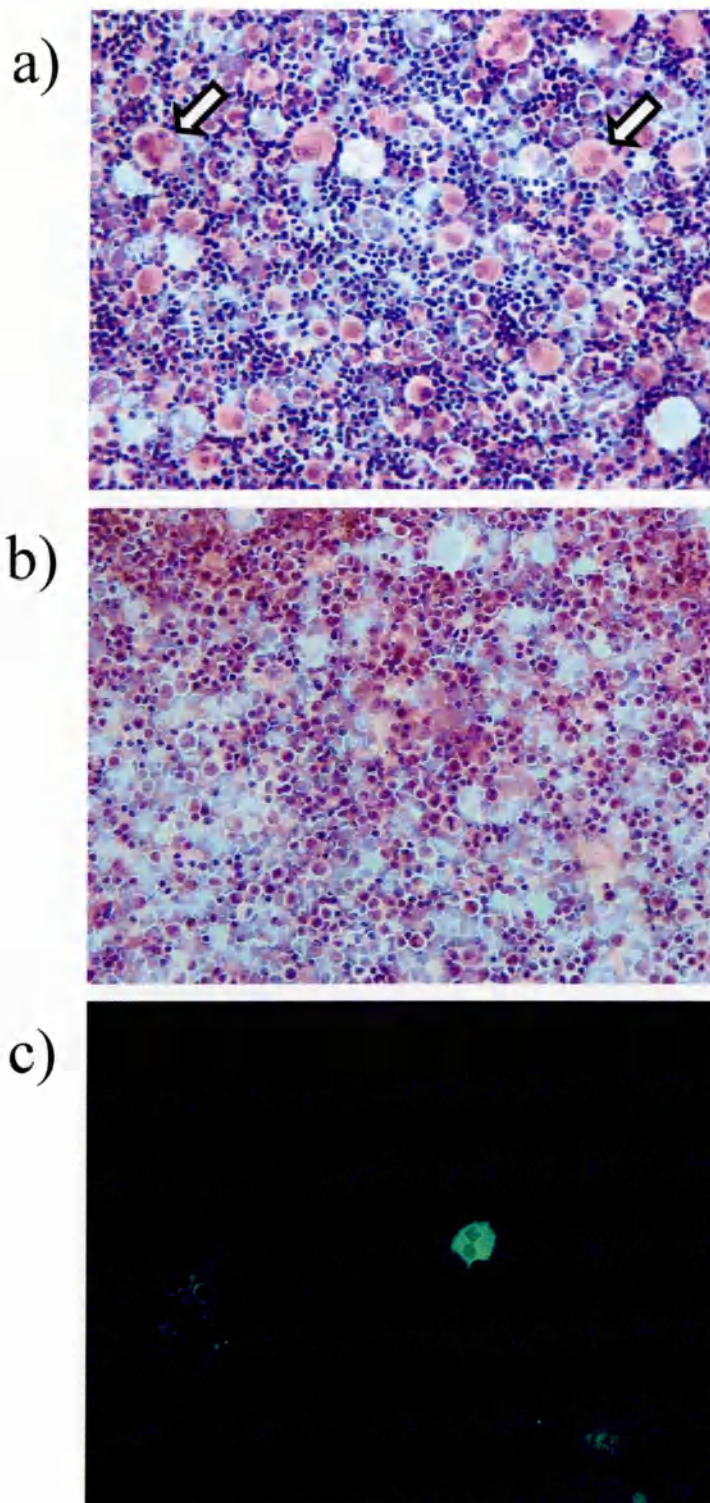
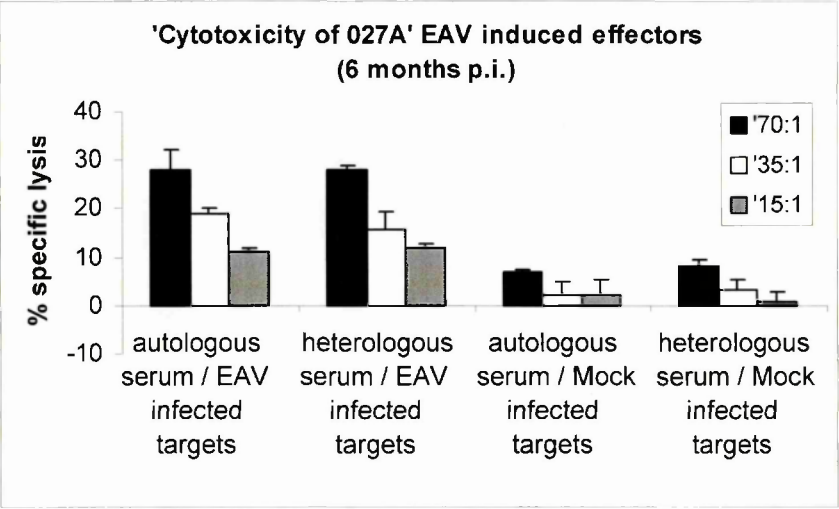


Figure 4.7. Features of in vitro stimulated PBMC cultures. Hematoxylin and eosin staining of 7 day old PBMC induction cultures shows a significant proportion of monocytes in mock-induced cultures (a) (white arrows) which are absent in EAV-induced cultures (b). Detection by immunofluorescence of EAV nucleocapsid (N) antigen in a 24 hour-old EAV-induced PBMC culture (c).

a)



b)

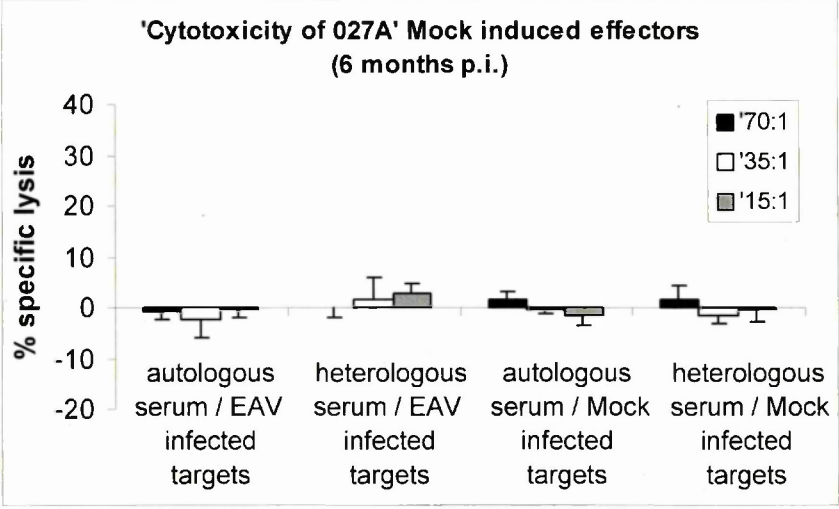
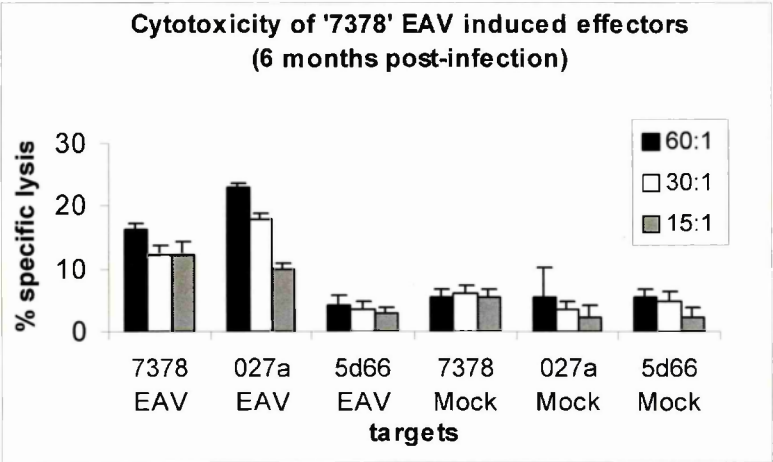


Figure 4.8. Effect of the source of serum (autologous vs heterologous) on the induction of EAV cytotoxic effectors. Effectors derived from PBMC of pony 027a were collected 6 months post-infection and the lytic activity was tested against EAV infected and mock infected autologous targets. The effector: target ratios are indicated in the boxes.

a)



b)

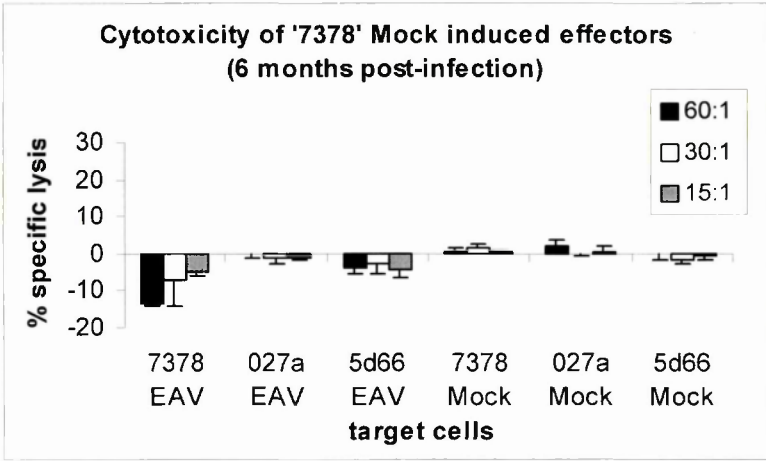
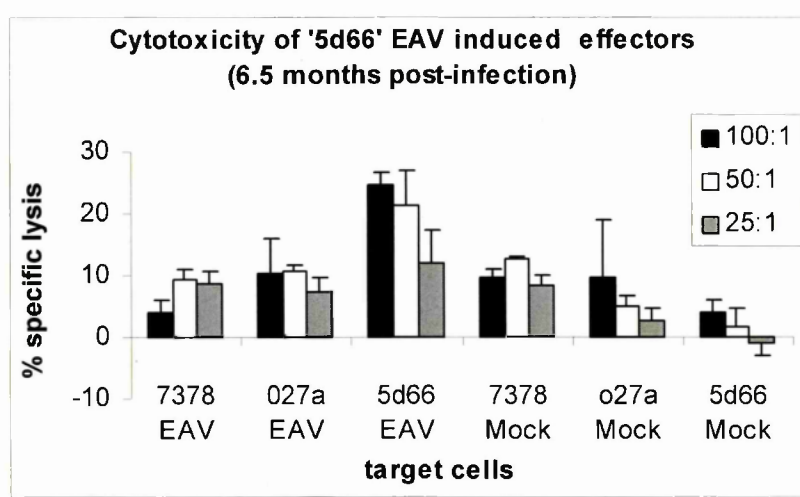


Figure 4.9. Genetic restriction of EAV specific cytotoxicity. Cytotoxic activity of EAV-induced and Mock-induced effectors derived from ponies 7378, 5d66 and 027a was tested against EAV-infected and Mock-infected autologous and allogeneic EDC targets. PBMC were collected from pony 7378 at 6 months post-infection (a and b), from pony 5d66 at 6.5 months post-infection (c and d) and from pony 027a at 8 months post-infection (e and f). Effector: target (E:T) ratios used for each set of effectors are indicated in the figure.

c)



d)

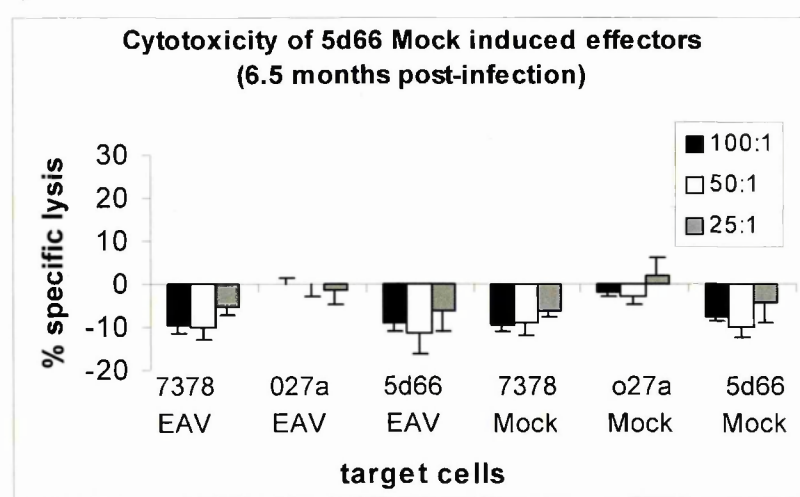
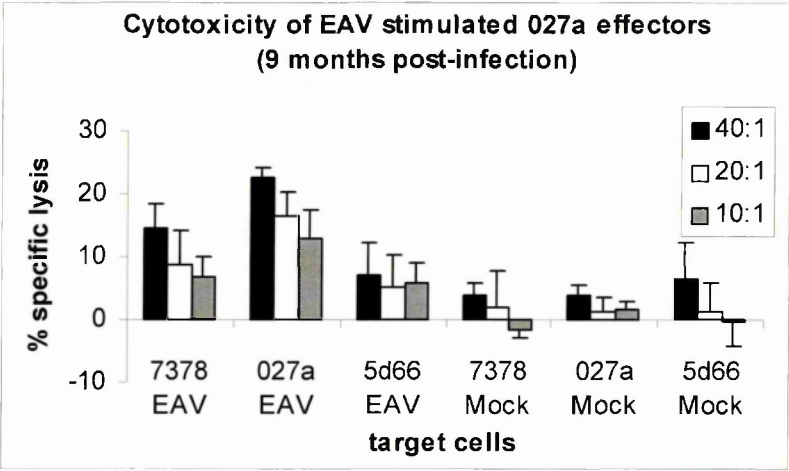


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e)



f)

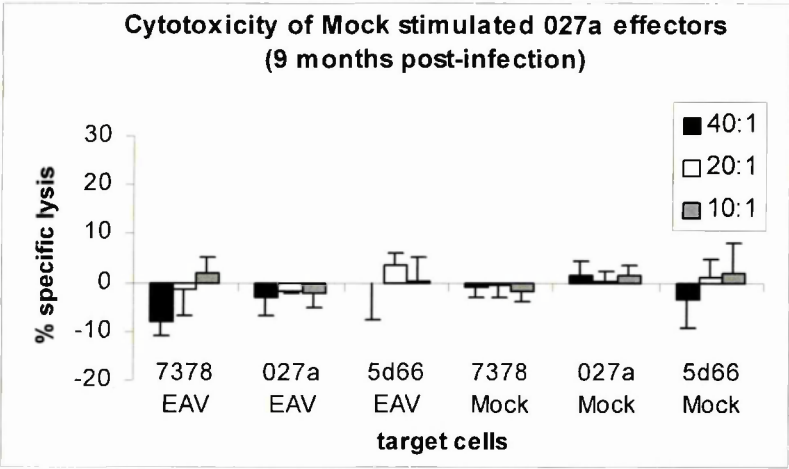
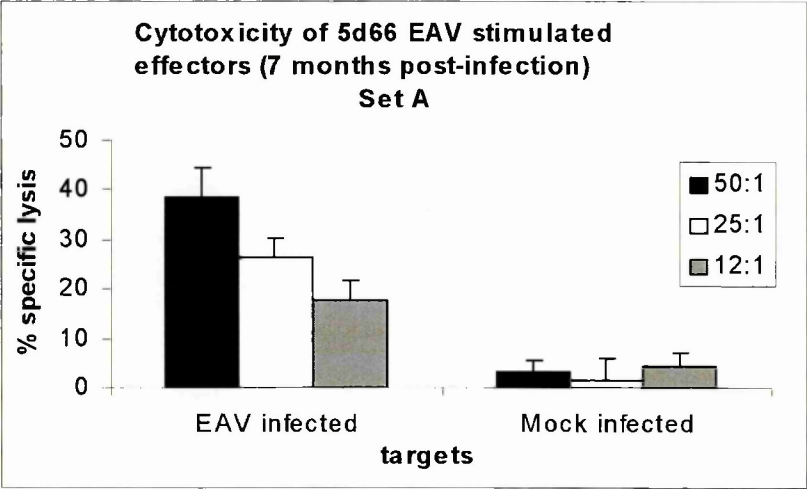


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a)



b)

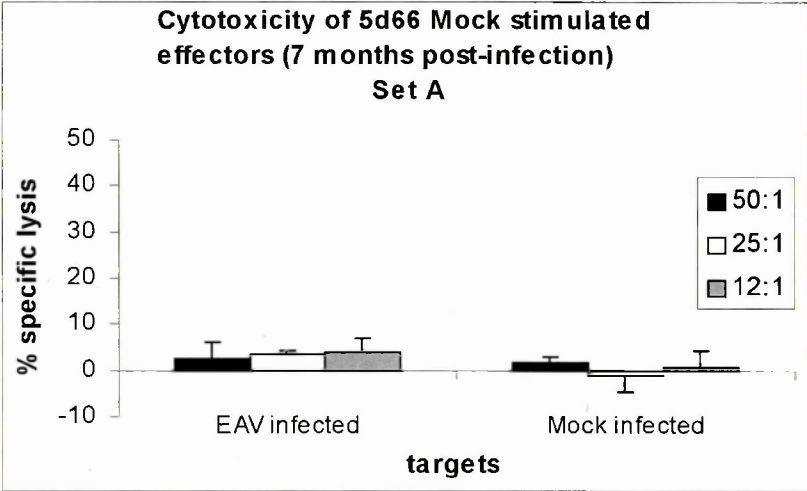
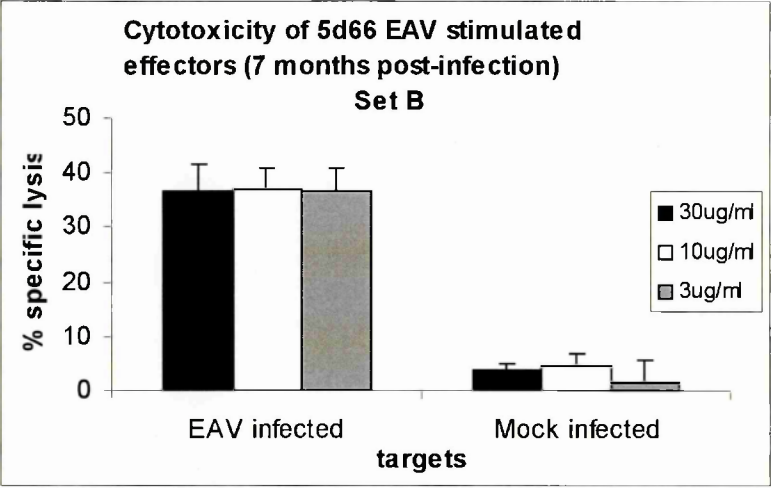


Figure 4.10. Anti-MHC-I MAb blocking experiment of EAV specific cytotoxicity. Effectors derived from pony 5d66 PBMC were incubated with EAV infected and Mock infected autologous targets in the absence (a, b) or presence (c, d) of different concentrations of anti-MHC-I MAb.

c)



d)

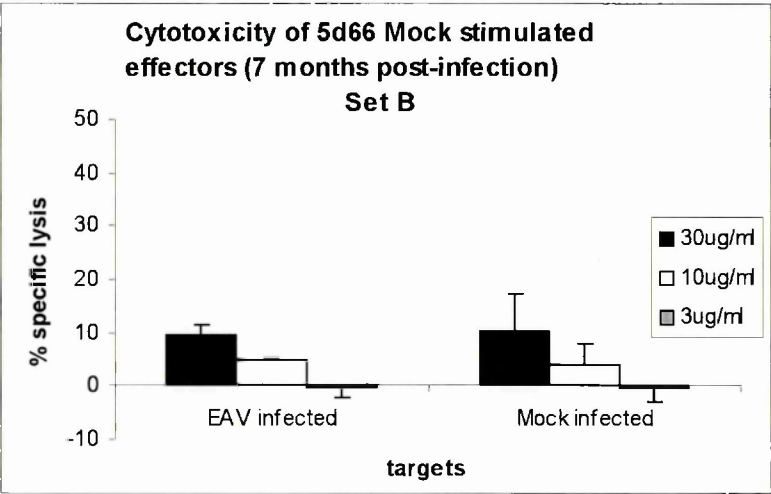
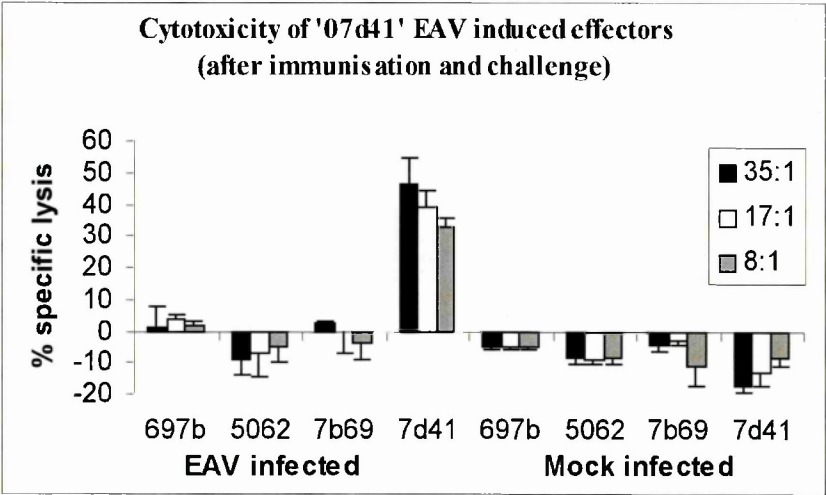


Figure 4.10. Anti-MHC-I MAb blocking experiment of EAV specific cytotoxicity. Effectors derived from pony 5d66 PBMC were incubated with EAV infected and Mock infected autologous targets in the absence (a, b) or presence (c, d) of different concentrations of anti-MHC-I MAb. E: T ratio 50: 1

a)



b)

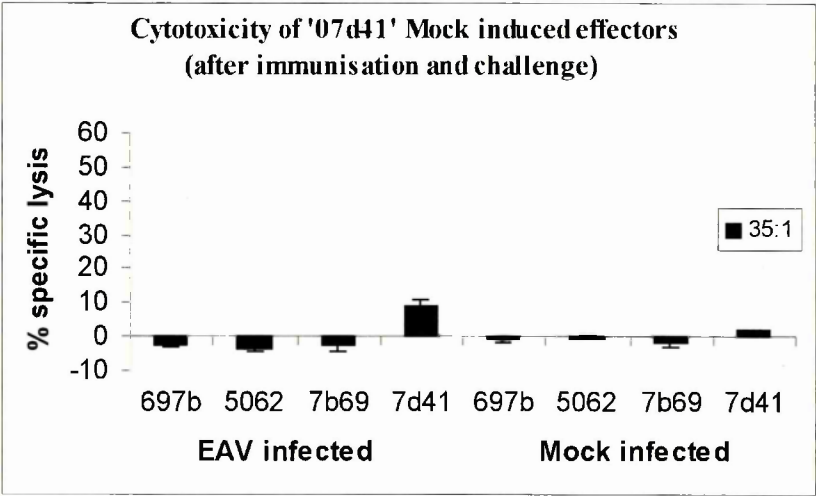
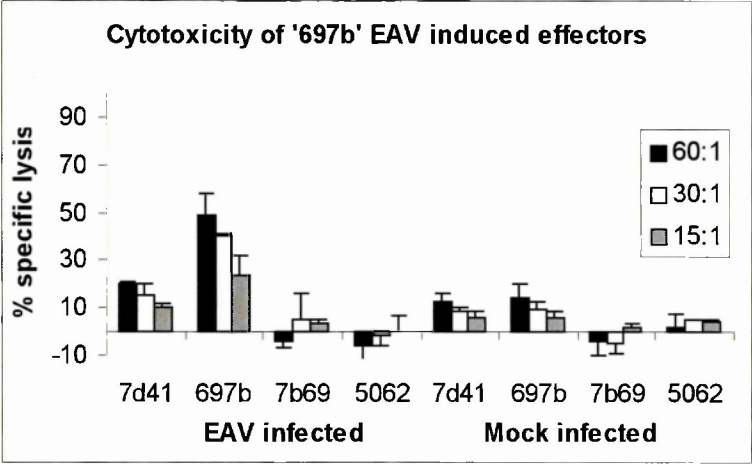


Figure 4.11. Genetic restriction of EAV specific cytotoxicity. EAV induced and mock induced effectors derived from pony 07d41 were incubated with EAV infected and mock infected autologous and allogeneic EDC targets.

a)



b)

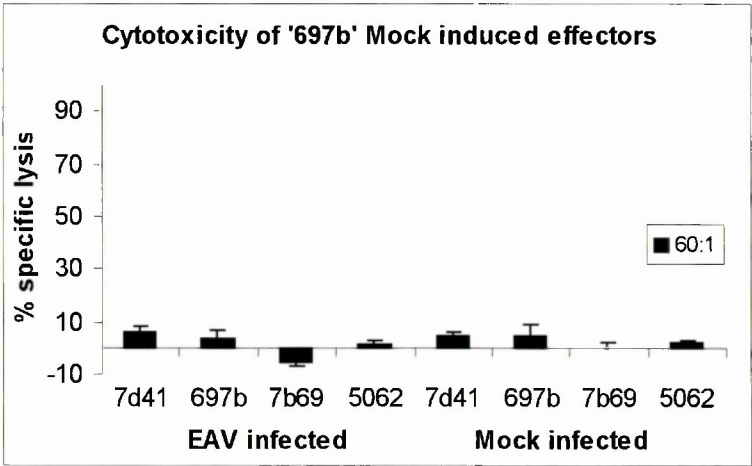
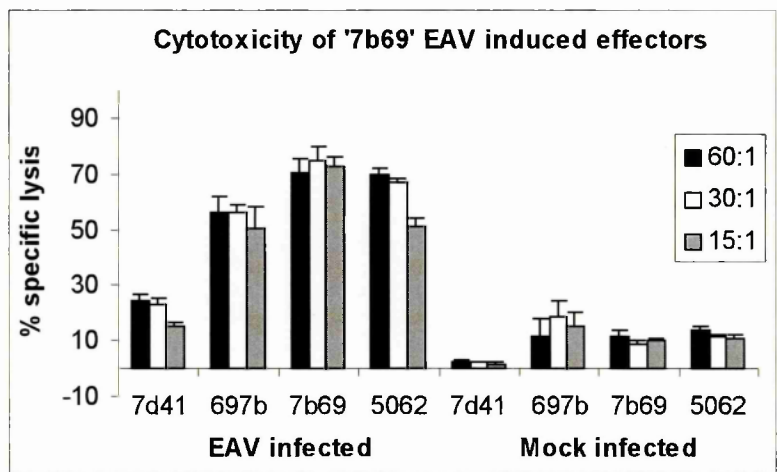


Figure 4.12. Genetic restriction of EAV specific cytotoxicity. Effectors derived from ponies 697b (a and c) and 7b69 (b and d) were tested against autologous and allogeneic EAV infected and mock infected EDC targets.

c)



d)

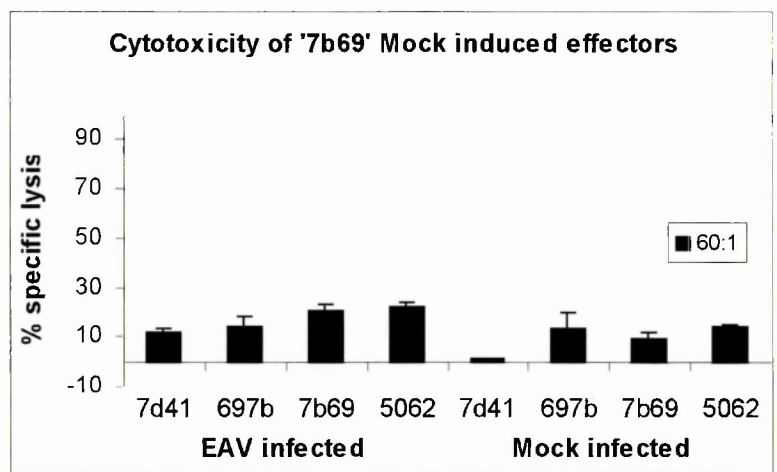
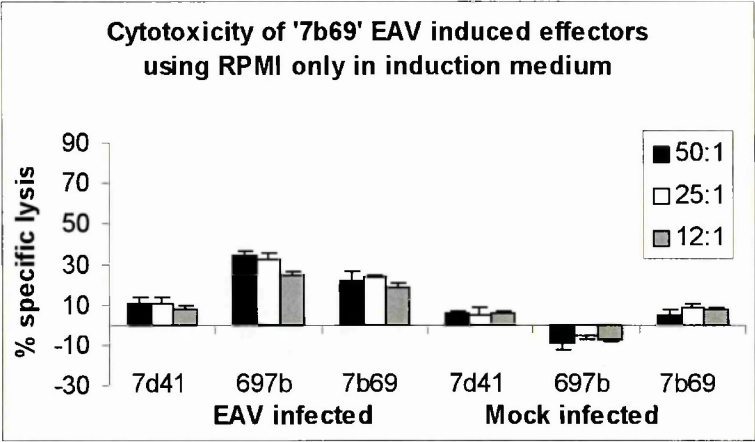
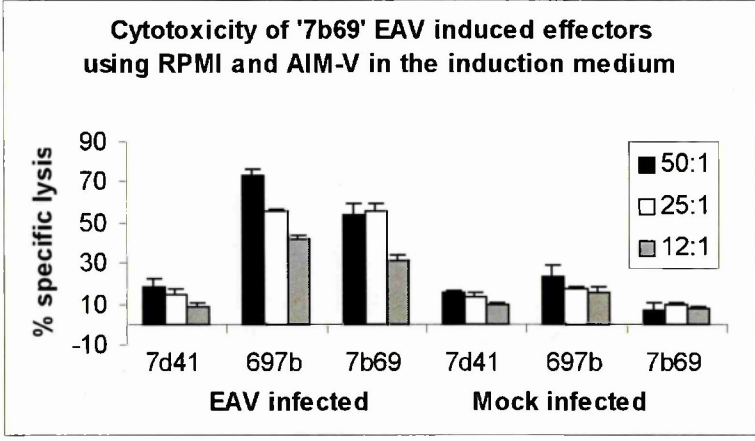


Figure 4.12. Genetic restriction of EAV specific cytotoxicity. Effectors derived from ponies 697b (a and c) and 7b69 (b and d) were tested against autologous and allogeneic EAV infected and mock infected EDC targets.

a)



b)



c)

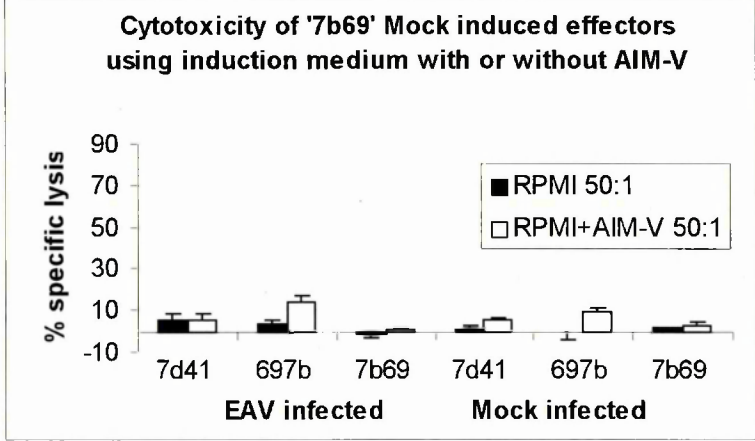
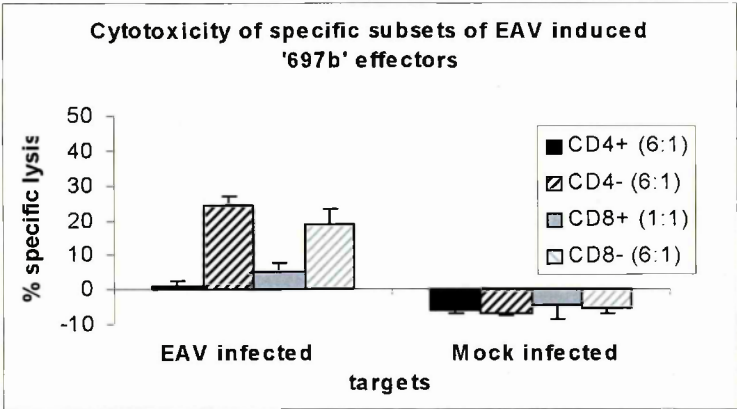
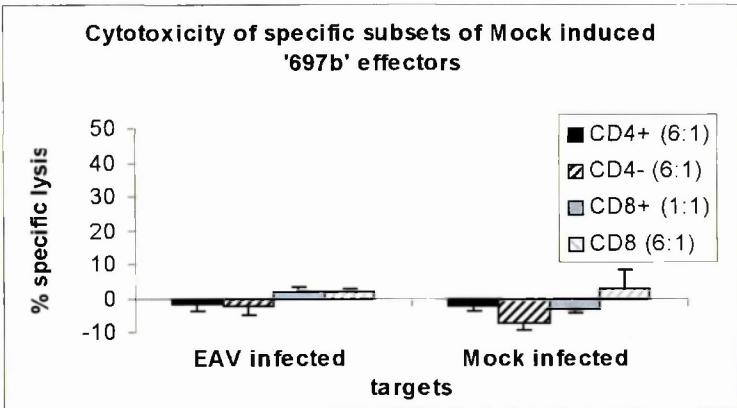


Figure 4.13. Effects of inclusion of AIM-V medium in the composition of the induction medium and assessment of genetic restriction of cytotoxicity. Cytotoxicity of EAV (a and b) and mock (c) induced effectors generated in induction medium with (b and c) or without AIM-V medium (a and c) is shown.

a)



b)



c)

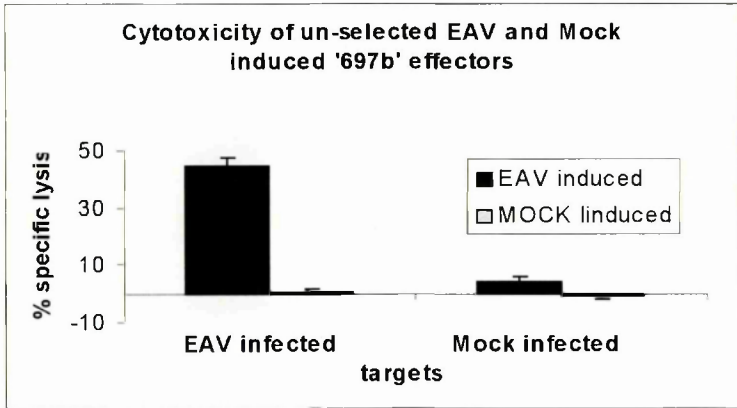
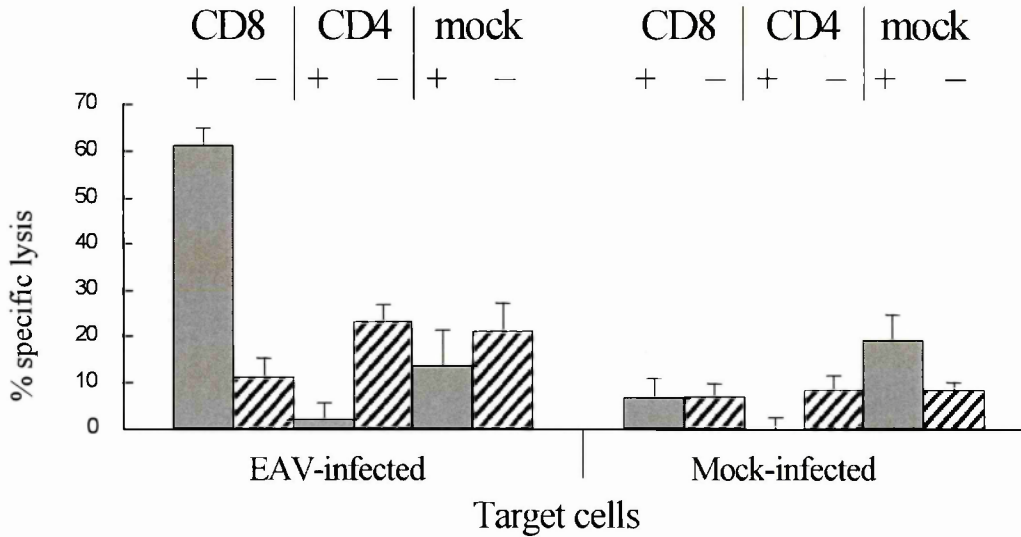


Figure 4.14. Phenotype of EAV specific effectors. EAV induced and mock induced effectors derived from pony 697b were processed for enrichment or depletion of CD8+ or CD4+ cells and tested against EAV infected or mock infected autologous EDC targets.

a) Fractionated effectors



b) Nonfractionated effectors

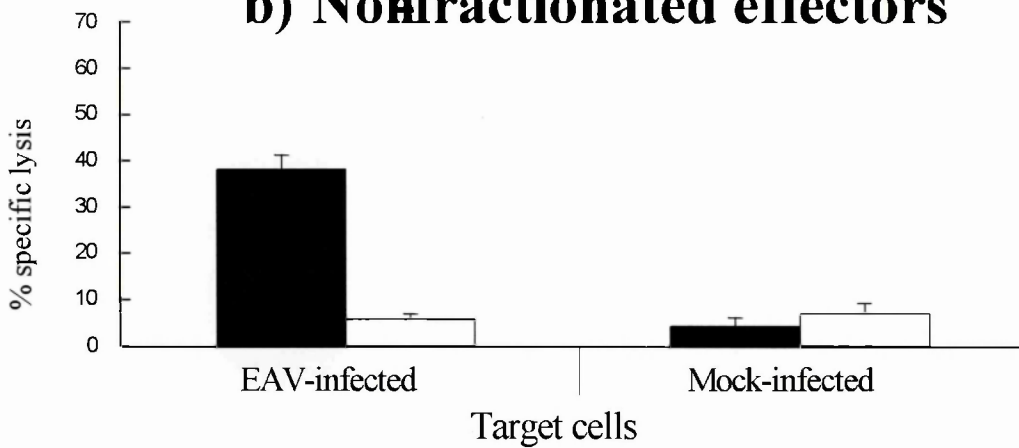


Figure 4.15. Phenotype of EAV specific cytotoxic effectors. Effectors derived from pony 697b (6 months post-infection) were tested for cytolytic activity against either EAV-infected or mock-infected autologous EDC targets. Cytolytic activity of fractionated EAV induced effectors, used at an effector: target ratio of 6:1 is shown in figure a). Effectors were either enriched for or depleted of specific cell-types, using MAb against CD8 or CD4. Buffer control samples were subject to the same separation procedures, but in the absence of Mab. Cytotoxic activity of non-fractionated effectors, used at an effector: target ratio of 25:1 is shown in figure b).

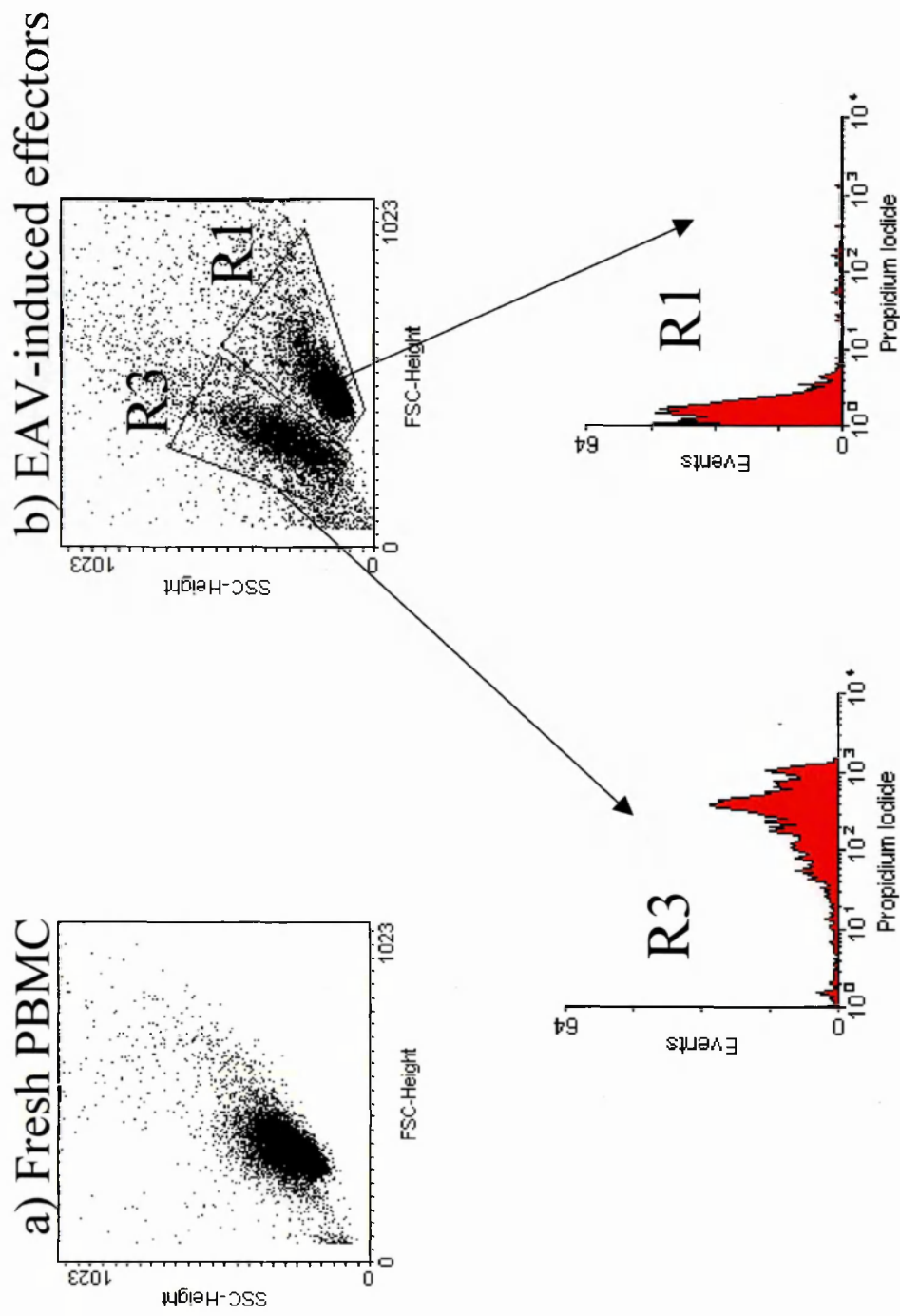
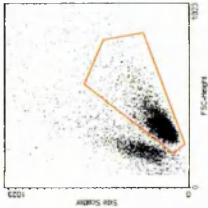


Figure 4.16. Flow cytometry analysis of EAV induced effectors. PBMC collected from pony 697b were analysed fresh (a) and after 7 days incubation in induction medium in the presence of EAV (b). EAV induced effectors were stained with propidium iodide to determine proportion of dead cells.

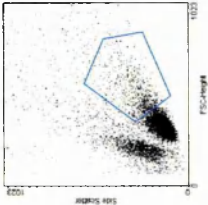
Figure 4.17. Flow cytometry analysis of 7 day old mock- or EAV-induced PBMC cultures derived from pony 5062 (PBMC collected one year after EAV infection). a)-d) Light scatter profiles, showing the regions gated for subsequent analyses [R1: a), c); R2: b), d)]. R1 corresponds to live cells as determined by propidium iodide exclusion (data not shown), R2 corresponds to activated lymphocytes (indicated by increased size and granularity). (e)-(l) Two samples from each culture were immunostained for detection of CD4 [panels e)-h)] or CD8 [panels i)-l)]. A third sample was immunostained with an IgG1 isotype control MAb (open histograms, overlaid on each of the CD4 and CD8 specific plots). For each histogram plot, the M1 region was analysed to determine the proportion of live cells (R1 gate) expressing either CD4 or CD8. Histograms display analysis of cells within either the R1 gate [panels e), i), g), k)] or the R2 gate [panels f), j), h), l)]. The number of cells counted within each region analysed is displayed in parentheses.

a)



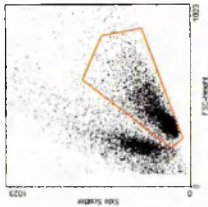
R1 = 75% total
(7472)

b)



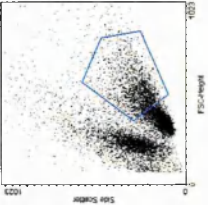
R2 = 12% R1
(876)

c)



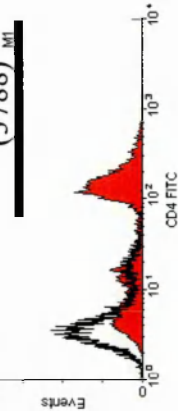
R1 = 56% total
(5580)

d)



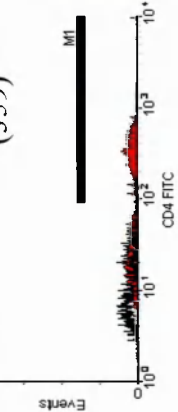
R2 = 39% R1
(2161)

e)



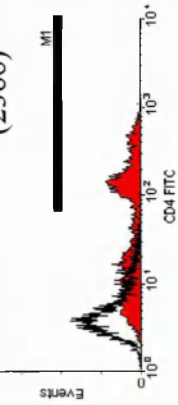
CD4+ = 51% R1
(3788)

f)



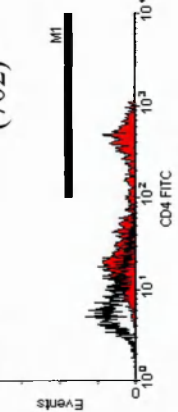
CD4+ = 5 % R2
(359)

g)



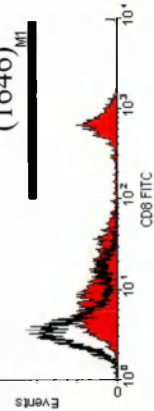
CD4+ = 43 % R1
(2366)

h)



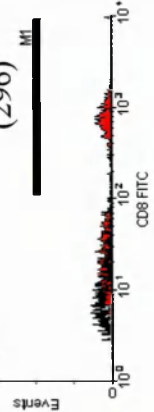
CD4+ = 13 % R2
(702)

i)



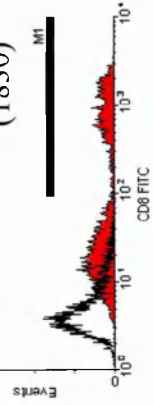
CD8+ = 22 % R1
(1646)

j)



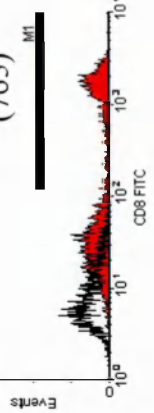
CD8+ = 4 % R2
(296)

k)



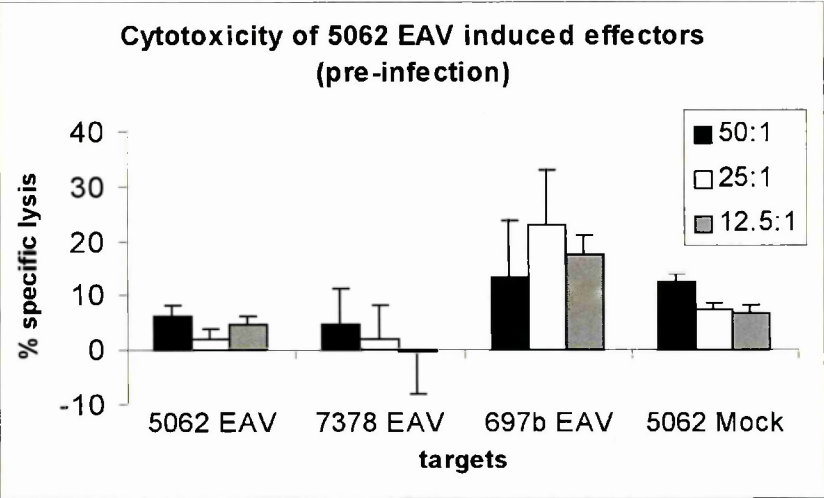
CD8+ = 33 % R1
(1830)

l)



CD8+ = 14 % R2
(765)

a)



b)

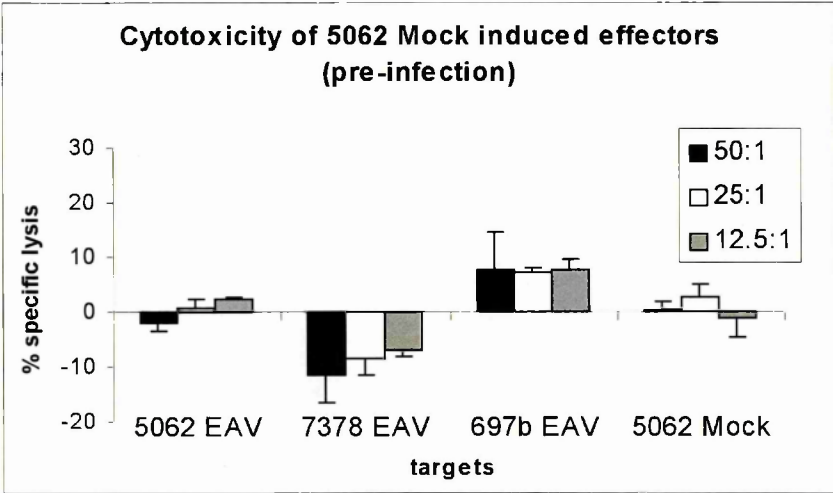
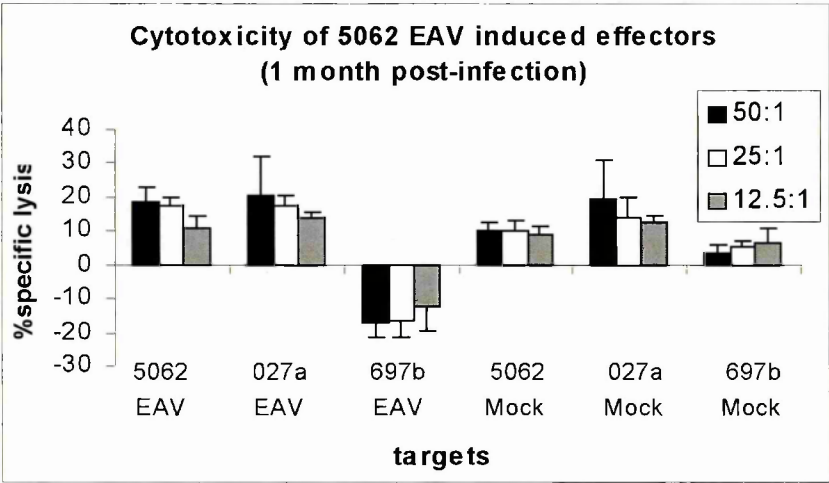


Figure 4.18. EAV specific cytotoxic activity of effectors derived from PBMC collected before, 1 month and 3 months post-infection against EAV infected and mock infected autologous and allogeneic EDC targets.

c)



d)

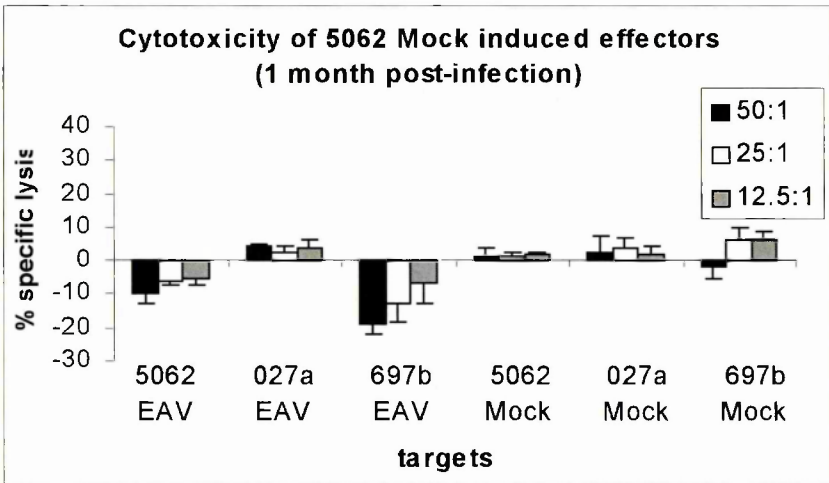
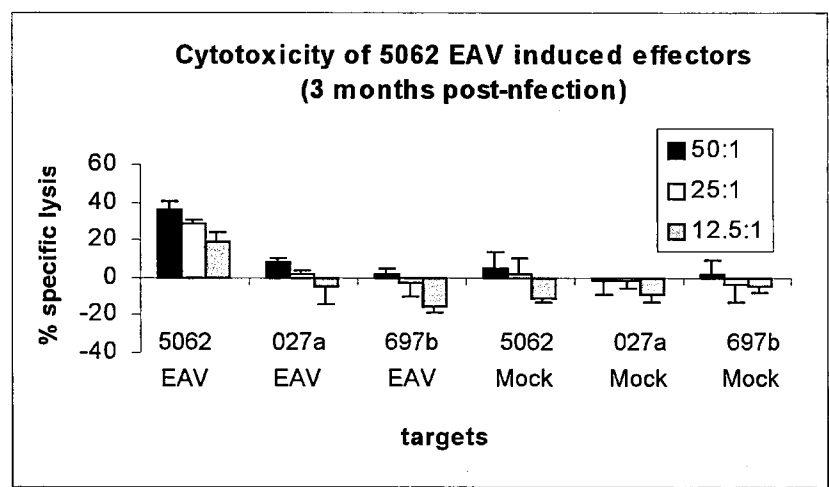


Figure 4.18. EAV specific cytotoxic activity of effectors derived from PBMC collected before, 1 month and 3 months post-infection against EAV infected and mock infected autologous and allogeneic EDC targets.

e)



f)

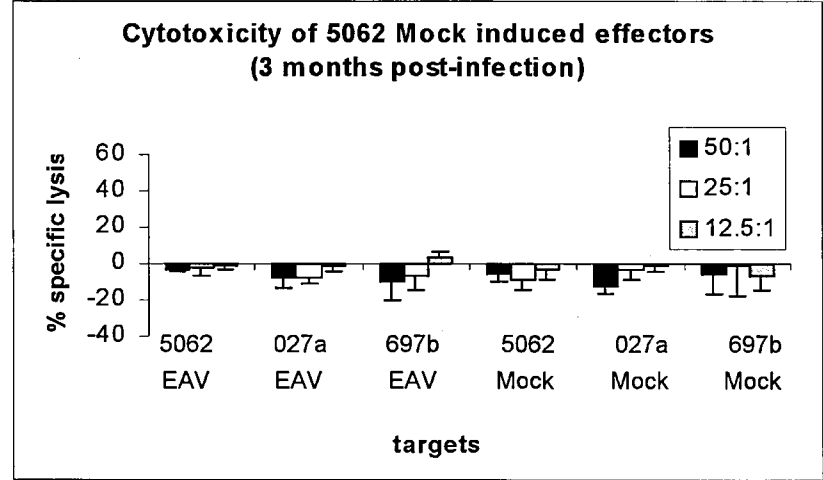


Figure 4.18. EAV specific cytotoxic activity of effectors derived from PBMC collected before, 1 month and 3 months post-infection against EAV infected and mock infected autologous and allogeneic EDC targets.

Chapter 5: Expression of single EAV genes in mammalian cells for identification of viral proteins containing CTL epitopes.

5.1. Introduction

The detection of cell mediated cytotoxic T-lymphocyte responses following EAV infection, and the solid immunity conferred by EAV-G_LΔ in the absence of a strong VNAb response suggest cellular immunity may play an important role in clearing virus infection. Therefore, the induction of CTL responses could be desirable or even necessary for the development of efficient EAV vaccines, an important consideration for vaccines based on single virus genes or their products. Identification of viral proteins containing CTL epitopes would then be required. The EAV CTL assay developed in this project (Chapter 4) will be adapted for this purpose. Single EAV genes will be expressed in EDC and incubated with EAV-specific cytotoxic effector cells and the levels of target lysis determined.

A plethora of methods and vectors have been used over the last decade by researchers for expression of heterologous genes in mammalian cells, ranging from plasmid DNA transfection to live recombinant viral vectors (Makrides, 1999). Viral vector expression systems such as vaccinia (Hammond *et al.*, 1998; McMichael *et al.*, 1986) or retroviral vectors (Lonning *et al.*, 1999) have been used to generate CTL targets to identify proteins harbouring CTL epitopes. Alternatively, other investigators identified viral proteins important for the

generation of CTL's using plasmid transfection or vaccinia recombinants to generate antigen presenting cells *in vitro* (Rouse *et al.*, 1994; Hammond *et al.*, 1998).

The purpose of the work presented in this chapter was to investigate methods for the identification of target proteins for EAV specific CTL's. Antigens derived from PRRSV ORF's 2, 5 and 6 have been recognised by pig T-lymphocytes in lymphoproliferation and delayed-type hypersensitivity assays (Bautista *et al.*, 1999). However, the only information on the antigenic specificity of the CTL responses during arterivirus infections comes from the studies of Even *et al.* (1995) demonstrating that 3T3 cells expressing the nucleocapsid protein of LDV (N/VP-1) stimulated the proliferation *in vitro* of LDV-specific CTL's. Thus, the N protein of EAV, encoded by ORF7 was chosen as the primary target for investigation. To generate EAV CTL targets, EAVORF7 was cloned into a mammalian expression plasmid vector and delivered into EDC by a non-liposome lipid transfection technique. Alternatively, baculovirus transduction was used. The system based on the *Autographa Californica* nucleopolyhedrosis baculovirus has been extensively used for the expression of recombinant proteins in insect cells (Galleno and Sick, 1999). Baculoviruses also have the capacity to transduce mammalian cells, i.e. delivering their genetic material into the cells without viral replication. Various reporter genes have been expressed in mammalian cells using this technique (Hofmann *et al.*, 1995; Boyce and Bucher, 1996; Shoji *et al.*, 1997; Aoki *et al.*, 1999). Attempts will be made to generate recombinant baculoviruses encoding EAV ORF7 downstream of a mammalian promotor for expression of the virus gene in mammalian cells.

5. 2. Materials and methods

5.2.1. Polymerase chain reaction (PCR).

Amplification of DNA by PCR for analysis of constructs and for cloning was performed with AmpliTaq DNA polymerase (Perkin Elmer Applied Biosystems) and Pwo DNA polymerase (Boehringer Mannheim, Germany) respectively. PCR's with AmpliTaq were typically performed in reactions containing 10mM Tris-HCl, 1.5 mM MgCl₂, 50mM KCl, 0.2 mM desoxyribonucleotides (dNTP's), 0.4 µM of each primer and 0.025 units of the enzyme. Reactions with Pwo DNA polymerase were performed in 10mM Tris-HCl, 25 mM KCl, 5 mM (NH₄)₂SO₄, 2.5 mM MgSO₄, 0.5 µM of each primer, 0.08mM of dNTP's and 0.05 units of enzyme.

The reactions were amplified in a DNA engine Tetrad Thermal cycler (M.J. Research Inc.). Cycles typically comprised the following steps:

- 1) DNA denaturation phase at 94°C for 4 min.
- 2) 30-40 cycles of:
 - a. denaturation step at 94°C for 45-60 seconds
 - b. primer annealing step at 45 – 60 °C for 60 seconds
 - c. extension step at 70 –72 °C for 90 to120 seconds
- 3) a final extension step at 70 – 72 °C for 5 minutes.

The number of cycles and the length and temperature of each step varied depending on the melting temperature (T_m) of the primers (annealing temperature usually 5 °C below primer T_m) and length of the DNA target.

The primers were obtained from Genset (France) and working stocks were prepared in sterile MQ water to 4 pmol / µl.

5.2.2. Electrophoresis of DNA on agarose gels

Agarose gels (0.7 % - 1.2 %) were prepared in TAE buffer (40mM Tris, 20mM acetate, 2mM EDTA) containing 5 µg / ml of Ethidium Bromide. DNA samples were diluted in loading buffer, 10-15 µl added to each well and the gel run at 70-120 volts for 30 to 60 min. A DNA molecular weight marker (1kb DNA ladder, Gibco Life Technologies) was included in each assay.

TAE buffer was prepared as 50x concentrated solution: 2M Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0.

Loading buffer was prepared as a 6x concentration solution: 0.25% Bromophenol Blue, 40% sucrose, 20mM EDTA.

5.2.3. Purification of DNA from solutions and from gels.

PCR products and plasmids were purified taking advantage of the selective binding properties of DNA to silica-gel in the presence of chaotropic salts at low pH. For these procedures the commercially available QIAquick Nucleotide Removal and QIAquick Gel Extraction kits from QIAGEN (Germany) were used.

After DNA separation by electrophoresis, the desired DNA fragment was sliced from the gel. Typically 400 mg were solubilised in buffer QG at 50°C. To ensure the pH is adequate for binding of the DNA to the silica membrane columns, 10 µl of 3M sodium acetate was added. The DNA sample was added to silica gel membrane spin mini-columns and these were centrifuged at 13000 rpm in an eppendorf microfuge for 1 min. The columns were washed twice in the ethanol containing buffer PE. The DNA was eluted by adding 50 µl of sterile MQ water to the column and centrifuging 1 minute later for 1 min at 13000 rpm.

DNA fragments and plasmids in solution were purified in the same way with the exception that 10 volumes of buffer PN were added to the DNA sample and centrifuged at 6000 rpm in the initial steps. Washing and elution steps were performed as above.

5.2.4. Adding adenosine and thymidine residues to the 3' ends of DNA ('tailing').

Purified DNA fragments were modified in their 3' ends by the action of AmpliTaq DNA polymerase in PCR reactions under normal buffering conditions but in the presence of only one of the dNTP's: 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl and, 200 µM dATP or 2 mM dTTP and 5 units of AmpliTaq. The reaction was incubated at 70°C for 35 min.

5.2.5. Dephosphorylation of 5'-ends of DNA fragments

The Shrimp Alkaline Phosphatase (SAP) (Roche) was used in a reaction containing: 50mM Tris-HCl, 5 mM MgCl₂, and 0.01 enzyme / pmol of 5'-phosphorylated DNA ends. These were calculated according to the formula: (µg DNA / kb size of DNA) x 3.04. The reaction was incubated at 37°C for 1 hour and subsequent inactivation at 65°C for 15 minutes.

5.2.6. Restriction digestions

Enzymes and buffers for restriction digestions were obtained from Promega (Madison, USA). Enzymes were used typically at 5 units / µg of DNA at the recommended temperature for each specific enzyme. Single digests were performed in the buffer recommended by the manufacturer for that enzyme.

Double digestions were performed sequentially including a purification step of the digested DNA, using the nucleotide removal procedure detailed above, in between the two enzymatic reactions. Enzymes were inactivated at 70°C for 5 min. after digestion.

5.2.7. Ligation of plasmid vectors and inserts

Enzyme and buffer were obtained from Promega (Madison, USA). Ligation reactions were performed at 4°C overnight in 30 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 10 mM DTT and 1 mM ATP, using an insert: vector molar ratio of 3:1 using 1 Weiss unit of T4 DNA ligase for 100 ng of vector.

5.2.8. Production of competent E. coli DH10B cells

This was done following the method of Hanahan (1985). A bacterial colony was picked and inoculated into 10 mls of LB broth and grown overnight at 37°C. The following day 50 ml of LB broth were inoculated with 1 ml of the overnight culture and grown at 37°C until the optical density (O.D.) reached 0.3-0.5. The bacteria were incubated in ice for 10 min, spun at 2000 rpm for 5 min, the pellet resuspended in 25 ml of cold buffer STB (100 mM KCl, 50mM CaCl₂, 10 mM CH₃-COOK) and incubated at 4°C for 30 min. After pelleting the cells by 5 min centrifugation at 2000 rpm the bacteria were resuspended in 2 ml of cold buffer FTB (100 mM KCl, 50mM CaCl₂, 10 mM CH₃-COOK, 15% glycerol) aliquoted and stored at -70°C.

LB broth: 10 g Triptone (Oxoid), 5 g Yeast Extract (Oxoid), 5 g NaCl (BDH), 1 litre MQ water. The medium was autoclaved at 15 p.s.i. for 15 min, cooled down

to room temperature and stored in the dark until used. LBA broth contains 100 µg / ml of ampicillin.

LB agar plates were prepared by dissolving 10 g Triptone (Oxoid), 5 g Yeast Extract (Oxoid), 5 g NaCl (BDH) and 13.6 g Agar (Oxoid) in 1 litre MQ water. The medium was autoclaved at 15 p.s.i. for 15 min, cooled down and poured slowly onto petri dishes (15 mls / plate). LBA agar plates were prepared by pouring the LB agar onto petri dishes containing 15 µl of ampicillin (100 µg / µl).

Ampicillin stock stolution: 100 µg dissolved in sterile MQ water and stored at – 20°C.

5.2.9. Transformation of competent cells

One vial of competent *E coli* was thawed slowly by incubation in ice for 30 minutes. Approximately 10 ng of DNA were inoculated into 100 µl of bacteria and incubated in ice for 30 min. The bacteria were heated at 42°C in a water bath for 90 seconds and then added to 1 ml of LB broth without ampicillin pre-warmed at 37°C and incubated at this temperature for 1 hour. Fifty µl of the bacterial suspension was streaked onto an LBA agar plate and incubated overnight at 37°C. If the cells were transformed with vectors containing the α-peptide of the β-galatosidase gene (such as pGEM-T', Promega), the bacteria were streaked onto LBAG agar plates to allow 'blue-white' screening of transformants. LBAG agar plates were prepared by adding and spreading 40 µl of X-gal (50 mg/µl) and 100 µl of 0.1 M IPTG onto the surface of LBA agar plates 10 min before streaking the bacteria.

IPTG solution (0.1 M): 1.2 IPTG (Promega) dissolved in 50 ml sterile MQ water and stored at 4°C.

X-gal solution (50 mg / ml): 100 mg of X-gal (Promega) dissolved in 2 ml N,N'-dimethylformamide (Sigma). Stored at -20°C.

5.2.10. Plasmid preparations

Plasmid DNA was isolated and purified from *E coli* cultures by alkaline lysis and binding of DNA to silica at high salt concentrations. The commercially available QIAprep (QIAGEN, Germany) was used for this purpose. Five ml of an *E coli* overnight culture in LBA broth were pelleted by centrifugation and resuspended in 250 µl of the RNase containing buffer P1. Addition of 250 µl of buffer P2 containing the detergent SDS and NaOH produced lysis of the bacteria and denaturing of the chromosomal DNA at the same time of releasing the plasmid DNA. Neutralisation of the reaction and adjusting the salt concentration with 350 µl of buffer N3 causes the plasmid DNA to bind to the silica membrane filters of the spin columns to which the samples were added after removing the precipitate by centrifugation for 10 min at 13000 rpm. The minicolumns were centrifuged, the flow-through removed and the columns washed twice with 0.5 ml of buffer PB by centrifugation at 13000rpm for 1 min. After removing the residual PE buffer by centrifuging for an extra minute the DNA was eluted by incubating the membranes with 50 µl of sterile MQ water for 1 minute and centrifugation for 1 minute at 13000 rpm.

5.2.11. Sequencing

Sequencing was performed using the dRhodamine Terminator Cycle Sequencing kit (Perkin Elmer Applied Biosystems). The dichlororhodamine dye dideoxynucleotide terminators, dNTP's, MgCl₂, AmpliTaq FS polymerase and buffer were provided in a single solution which was then used (4µl) with 3 µl of DNA (0.03 – 0.2 µg / µl), 0.4 µl of primer (4 pmol/µl) and 2.6 µl of sterile MQ water. The reaction was subjected to 25 cycles of:

- 1) 96°C, 30 seconds
- 2) 50°C, 15 seconds
- 3) 60°C, 4 min

followed by a final incubation step at 4°C.

The DNA (10 µl) was finally precipitated by adding a 95% ethanol solution (25 µl) and 3M sodium acetate (1 µl) and incubating the mixture for 7 min on ice. After centrifuging at 13000 rpm for 15 min, the pelleted DNA was resuspended in 70% ethanol (125 µl) and centrifuged again at 13000 rpm for 10 min. The DNA pellet was then dried out and resuspended in 10 µl of Hi-Di Formamide before loading the samples into the ABI Prism 3700 DNA Analyser.

5.2.12. Tissue culture techniques

Mammalian primary (EDC) and continuous cell lines (RK-13, COS-7 cells) and the insect cell line Sf9 were used through the study.

RK-13 cells and EDC's were obtained and cultured as described previously (Chapters 2 and 4 respectively). COS-7 cells were obtained from the European Collection of Cell Cultures (ECACC). This cell line was grown in 10% MEM in

75 cm² tissue culture flasks and passaged routinely once a week using a splitting ratio of 1:3.

The insect cell line Sf9 was obtained from Gibco-Life Technologies and grown in insect cell growth medium [TNM-FH medium (Sigma) supplemented with 10% heat inactivated bovine serum (Gibco Life Technologies) and 50 IU/ml penicillin and 50µg/ml streptomycin (Gibco Life Technologies)]. Cells were cultured in 75 cm² tissue culture flasks as monolayers which were split 1:3 weekly by replacing the medium and resuspending the cells in fresh insect cell growth medium.

5.2.13. Transfection of plasmid DNA

Transfections of plasmids into EDC's, COS-7 and RK-13 cells were performed using the non-liposomal lipid reagent FuGENE (Boehringer Mannheim). The reagent was added to a small volume of SFMEM [minimum essential medium Eagle with Earle's salts (Sigma, M2279) supplemented with 100 IU of penicillin per ml, 10 µg of streptomycin per ml, non-essential amino acids, L-glutamine to a final concentration of 2 ml] and incubated for 5 min at room temperature before adding it to the plasmid preparations. The mixture was incubated for 15 min at room temperature and added slowly and evenly to 80 % confluent cell sheets, previously washed in SFMEM. After addition of 10% MEM the cells were incubated at 37°C without removing the reagent.

Insect cells were transfected using the cationic liposome reagent Lipofectin (Invitrogen) . Cells were seeded onto 6 well flat bottomed plates at a density of 10⁶ cells / well and incubated at 28°C for 24 hours in insect cell growth medium. Then the cell sheets were carefully washed with serum free insect cell growth

medium and 1.5 ml of this medium was added to each well. A volume of 50 μ l containing linearised baculoviral DNA and the plasmid was mixed with 50 μ l of a solution containing 100 μ g / ml of lipofectin transfection reagent. This mixture was incubated for 20 min at room temperature and then added to the cells. The cells were incubated further for 5 hours at 28°C after which 1.5 mls of insect cell growth medium were added and the incubation continued for 4 days.

5.2.14. Detection of expression of recombinant genes in mammalian cells

The reporter gene *lacZ* was detected in EDC's, RK-13 cells or COS-7 cells by staining the cells with a 0.2 % X-Gal solution prepared by diluting an X-Gal stock solution (2% X-Gal, dymethyl-formamide) in 2mM MgCl_2 , 5mM $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$, 5mM $\text{K}_3\text{Fe}(\text{CN})_6$. Cells were washed with PBS after removing the medium, fixed with 0.25% glutaraldehyde in PBS, washed again with PBS and incubated with the X-Gal solution overnight. After removing the X-Gal solution the cells were covered with PBS and observed for the development of blue colour.

EAV protein expression was detected by indirect immunofluorescence. Cells were washed with PBS before being fixed with 0.4% Triton-X-100 – 4% formaldehyde in PBS or 4% formaldehyde in PBS. After removing the fixing solution and washing with PBS, a 1/100 dilution of anti-N specific rabbit polyclonal antibody α -SP07 (de Vries *et al.*, 1992) in PBA (0.2% bovine serum albumin (Sigma) in PBS) was applied for 1 hour at 37°C. Finally, after washing the cells in PBS, FITC conjugated goat anti-rabbit IgG (Dako, Denmark) was added to the cells at a 1/40 dilution in PBA for 1 hour at 37°C before a final

washing step was performed and cells observed on a Leica microscope under UV light.

5.2.15. Baculovirus plaque assay

Dilutions of recombinant baculovirus and a control BacPak6 baculovirus were absorbed (100 µl) to Sf9 insect cell confluent monolayers prepared on 6 well flat bottomed plates and incubated for 1 hour at 20°C. Then they were replaced by 2 mls of a 1 % agarose overlay [1:1 insect cell growth medium, 2 % low-melting point agarose (Sigma) in water]. Once the agarose set, 2 mls of fresh insect cell growth medium was added and the plates incubated at 28 °C for 3 days. The medium was removed and 1.5 mls of a neutral red solution [0.025 % neutral red (Sigma), PBS] was added to each well and incubated for 3 hours. Then the stain was aspirated and plates were inverted and incubated overnight at room temperature. Pale pink plaques were observed in a red background and were examined under the microscope to check that they consisted of insect cells which did not uptake the stain. Several plaques were picked by aspirating the agarose with a sterile pasteur pippette and transferred to 0.5 mls of serum free insect cell medium and stored at 4°C until used. Each plaque pick was used to infect a fresh cell monolayer. After a 3 day incubation period the tissue culture fluid was harvested and stored at 4°C while the cell sheets were washed with PBS. After fixing the cells with 0.25% glutaraldehyde and washing twice in PBS the cells were incubated with 1 ml of medium containg 0.002 % X-Gal for 2 hours to check that the recombinant viral plaques did not contain BacPak6. BacPak6 infected cells, included as a control in the assay, developed a blue colour in contrast to the recombinant baculovirus infected cells.

5. 3. Results

5.3.1. Expression of EAV ORF7 by plasmid transfection.

The multipurpose mammalian cell expression vector pcDNA3 (Invitrogen) was chosen for expression of EAV ORF7 in EDC. Apart from the conventional prokaryotic origin of replication and selection markers (colE1 and ampicillin resistance gene respectively) which allow plasmid propagation in bacteria, this vector has a multiple cloning site flanked upstream by a human cytomegalovirus immediate-early promoter (CMV) and phage T7 promoter and downstream by a bovine growth hormone polyadenylation site for protein expression in mammalian cells. In addition, it contains the simian virus 40 (SV40) origin of replication which allows the replication of the plasmid in COS-7 cells. At the time this work was performed, co-workers had experienced some difficulties in cloning different genes into this vector directly from PCR products but not by sub-cloning from other vectors. The strategy used, outlined in Fig. 5.1, involved cloning of ORF7 into an intermediate plasmid vector (pGEM-T', Promega) from an adenosine (A') 'tailed' PCR product, followed by sub-cloning into pcDNA3.

5.3.1.1. Cloning of EAV ORF7 into plasmid pGEM-T'.

Amplification of the EAV nucleocapsid gene was performed by PCR from cDNA106 (de Vries *et al.*, 1992) using primer 2510001 (5'-TAGGATCCACCATGGCGTCAAGACG-3') and primer 2510002 (5'-TAGGATCCTTACGGCCCTGCTG-3'). These primers contain *Bam*HI restriction sites for eventual insertion of the gene into the multiple cloning site of pcDNA3 (Fig. 5.2). A Kozak sequence (Kozak, 1989) was included in the forward primer 2510001 for efficient translation of m-RNA in mammalian cells.

To minimise the introduction of mutations during PCR, a proof reading (3'-5' exonuclease activity) thermostable DNA polymerase (*Pwo*, Boehringer Mannheim, Germany) was used in the PCR reaction. A band of approximately 330 nucleotides was visualised after running the PCR product in an ethidium bromide 0.9% agarose gel and was then purified. The *Pwo* enzyme, due to its 3'-5' exonuclease activity, generates blunt-ended PCR fragments and therefore the addition of adenosine to the 3'-ends is required for efficient ligation into the cloning plasmid vector pGEM-T'. This was achieved by incubating the purified ORF7 PCR product with AmpliTaq DNA polymerase in the presence of dATP. In these conditions, the AmpliTaq DNA polymerase incorporates single A' residues to the 3' terminal ends of double stranded DNA. Once the reaction was completed, the modified ORF7 PCR product was purified from enzyme, buffer and salts and resuspended in water. Then it was inserted into the T'-tailed pGEM-T vector and transformed into DH10B *E coli* and incubated on LBAG plates overnight. The 3' T overhangs in the pGEM-T' vector are positioned within the α -peptide sequence of the β -galactosidase gene. Insertional inactivation of the enzymatic activity allows visual screening of recombinant clones which appear white in LBAG agar plates. Four white bacterial colonies and one blue colony were selected, resuspended in sterile water and heated at 95°C for 4 minutes to lyse the cells. The presence in the lysates of the recombinant plasmid was checked by PCR with the EAVORF7 specific primers 250001 and 250002. A band of the expected size was obtained from samples of the four white clones and small plasmid preparations were made of each of these clones. Restriction digestions of the plasmid preparations with *Bam*HI showed fragments of approximately 3000 base pairs and 330 base pairs confirming the

presence of EAV ORF7 in the pGEM-T plasmid. Sequencing of the insert using primers M13+ (5'-GTTTTCCTCCAGTCACGAC-3') and M13- (5'-CAGGAAACAGCTATGAC-3'), which flank the multiple cloning site of pGEM-T vector, confirmed the presence of the EAV ORF7 gene insert in the plasmid preserving the *Bam*HI restriction sites. One of these recombinant plasmids was selected and denoted pGEM-T / ORF7.

5.3.1.2. Subcloning of EAV ORF7 into pcDNA3

For subcloning into pcDNA 3, plasmid pGEM-T / ORF7 was digested overnight at 37°C with *Bam*HI and the enzyme was subsequently inactivated by heat and the reaction run on a 0.9 % agarose gel. The 330 base pairs fragment was purified from the gel and ligated into pcDNA3 digested with *Bam*HI and dephosphorylated at its 3'ends. The ligation reaction was transformed into competent DH10B cells and these were plated out onto LBA plates. Seven bacterial clones were checked for the presence of the recombinant plasmid by PCR using ORF7 specific primers 2510001 and 2510002. Amplicons from five of the clones produced a positive result (Fig.5.2). To check the orientation of the insert in the plasmid , a new PCR reaction was prepared using primer 2510001 and primer CMV seq (r) (5'-GAAGGCACAGTCGAGGCT-3'), specific for pcDNA3 and positioned downstream of the multiple cloning site in reverse orientation. Three clones contained the insert in the correct orientation in the recombinant plasmid, with the translation initiation codon downstream of the CMV / T7 promoter elements. Large scale plasmid preparations were made for each plasmid. Sequencing PCR reactions using primers CMVseq (5'-ATAAGCAGAGCTCTCTGGC-3') and CMVseq(r) confirmed that the ORF7

sequence, the insert-vector junctions and orientation were correct for one clone 3c. This recombinant plasmid was denoted pcDNA3 / ORF7.

5.3.1.3. Expression of EAV N in EDC by pcDNA3 / ORF7 plasmid transfection.

Preliminary experiments to test the efficiency of expression of recombinant proteins via a cationic lipid (FUGENE) mediated delivery of recombinant plasmids into EDC were conducted using the pcDNA3 / lacZ plasmid, made and supplied by Dr McAleer. This plasmid has the backbone of pcDNA3 into which the β -galactosidase gene was inserted downstream of the T7 / CMV promoter region for expression in mammalian cells.

An experiment was set up to compare the levels of protein expression between EDC and COS7 cells after plasmid transfection of pcDNA3 / lacZ. Subconfluent monolayers of COS7 cells and EDC prepared on 6 well plates (approximately 2×10^5 cells / well) were incubated for 15 minutes with 1 μ g of the plasmid prepared in a 100 μ l suspension of SFMEM containing 1.5 μ l of FUGENE. After 15 min incubation at room temperature, 1 ml of 10% MEM was added to each well and the plates were incubated for 24 hours before checking expression of the lacZ gene. The medium was discarded and cells washed twice with PBS before being fixed with glutaraldehyde for 15 minutes. Three washes with PBS preceded the addition of X-Gal solution which was incubated with the cells for three hours at 37°C. After this incubation period, 5 % of the EDC turned blue indicating poor efficiency of transfection in these cells, as opposed to COS7 cells which showed 50 % lacZ expression efficiency (data not shown).

A second experiment was performed to optimise the FUGENE mediated transfection of pcDNA3 plasmids in EDC. For this, two 24 well plates were prepared with 4 sets of EDC monolayers containing 2.5×10^4 , 5×10^4 , 10×10^4 or 20×10^4 cells per well. After 24 hours incubation at 37°C , each set of cells of each plate was incubated with 0.125, 0.250, 0.500 or 1 μg of pcDNA3 / lacZ prepared in 50 μl of SFMEM containing 1.5 μl of FUGENE. Then 1 ml / well of either 10% MEM or 20% MEM was added to the plates and these were incubated for a further 24 hours at 37°C . Expression levels of the reporter gene after X-gal staining was highest when the cells were seeded at 20×10^4 cells / well, transfected with 1 μg of plasmid and incubated in 20% MEM after transfection. However these levels were never higher than 10% (data not shown).

Expression from the pcDNA3 / ORF7 construct was tested on COS7 cells to verify the quality of the construct and determine the time of maximum expression after transfection. In this experiment, Vaccinia MVA / T7 co-infection was used to facilitate the expression of the EAV N gene. This recombinant virus encodes the RNA polymerase of phage T7, which once synthesized, provides cytoplasmic transcription of expression cassettes containing the CMV/T7 promoter elements such as the one contained in the pcDNA3 / ORF7 plasmid. Four 24-well plates were inoculated with 3×10^4 cells / well and incubated in 10% MEM for 24 hours before being tested. For each plate, the cells were either a) mock infected; b) infected with EAV at an m.o.i. of 0.8 TCID₅₀ / cell; c) infected with Vaccinia MVA / T7 at an moi of 10 pfu / cell; d) transfected with 240 ng of pcDNA3 / ORF7 per well or e) infected with Vaccinia MVA / T7 at an m.o.i. of 10 pfu / cell and transfected with 240 ng of

pcDNA3 / ORF7 per well. Cell monolayers were first inoculated with 250 µl of either SFMEM (a and d), EAV (b) or MVAT7 (c and e). Transfections were conducted using FUGENE: plasmid µg ratio of 12:1 (d and e). After 1 hour incubation all the wells were incubated in 10% MEM for various periods of time. The cells were fixed at 1 hour, 2.5 hours, 5 hours or 24 hours post-transfection. For each plate, one replicate was fixed with 4% formaldehyde whereas the other was fixed with 4% formaldehyde, 0.4% Triton-X-100. Detection of the EAV N antigen by immunofluorescence revealed ORF7 expression from EAV infected cells only at 24 hours-post inoculation and from cells simultaneously infected with vaccinia MVAT7 and transfected with pcDNA3 / ORF7 at 5 and 24 hours post-inoculation. An example of the immunostaining pattern obtained in pcDNA3 / ORF7 transfected and MVAT7 co-infected COS-7 cells fixed at 24 hours is shown in Figure 5.3. The use of 0.4 % Triton-X-100 to permeabilise the cells was necessary to detect an immunofluorescence signal. These experiments corroborated that the cloning of EAV ORF7 into the pcDNA3 expression vector was satisfactory and gave an indication about the time of maximum expression. However, RNA transcription from the cytoplasm, provided by the T7 RNA polymerase expressed from the recombinant vaccinia MVA /T7, was required for detectable expression of the N protein. This may indicate either that the plasmid was inefficient at entering the nucleus or that ORF7 mRNA transcripts in the nucleus were inefficiently translated (eg. RNA instability, low efficient export to the cytoplasm).

In order to test expression of ORF7 in EDC and RK-13 cells following transfection of pcDNA 3 / ORF7, EDC and RK-13 cells were seeded at 10^4 cells

/ well and incubated for 24 hours in 20% MEM and 10%MEM respectively before being transfected with 240 ng of either pcDNA3 / ORF7 or pcDNA3 / lacZ using FUGENE: µg plasmid ratios of 3:1, 6:1 and 12:1. Half of the wells receiving pcDNA 3 / ORF7 were co-infected with Vaccinia MVA T7. Positive controls were provided by EAV infected cells and negative controls provided by MVA / T7 infected and mock-infected cells. After an incubation period of 24 hours at 37°C, the cells were fixed with 4% formaldehyde, 0.4% Triton-X-100 solution and expression of the recombinant genes tested by immunofluorescence or X-gal staining. Results indicated that RK-13 cells expressed both recombinant genes and that the efficiency correlated with the FUGENE: plasmid ratio in the following order: 3:1 > 6:1 > 12:1. However, EDC's failed to express either recombinant gene efficiently (less than 5% cells expressing the recombinant gene). Intriguingly, expression of EAV N in RK-13 cells was observed in the presence and absence of MVA/T7 (data not shown).

5.3.2. Expression of heterologous genes in EDC by baculovirus transduction

5.3.2.1. Expression of lacZ in EDC by baculovirus transduction

At the time these experiments were being conducted, Dr McAleer at the AHT expressed successfully the β-galactosidase enzyme in COS7 cells and equine embryonic lung cells (EEL) by the use of a recombinant baculovirus (Bac-CMV / lac Z) which encodes the lacZ gene under the control of the CMV / T7 promoter. These results demonstrated the potential of baculovirus transduction for high efficiency expression of EAV genes in EDC. A preliminary experiment was set up to test the validity of this approach for expression of lacZ in these cells. Doubling dilutions of the Bac-CMV / lacZ baculovirus stock, used by Dr

McAleer, were made in SFMEM or in 20% MEM and 100 μ l of each dilution inoculated into each of four replicate wells of EDC monolayers seeded 24 hours earlier at 10^4 cells / well. Wells inoculated with baculovirus suspensions in SFMEM were replaced by fresh 20 % MEM after 4 hours incubation at 37°C and then incubated for a further 20 hours. The monolayers inoculated with the 20% MEM baculovirus suspension were incubated for 24 hours without medium change. After the incubation period, the cell sheets were stained with X-Gal. The results of this experiment showed: a) that heterologous expression of *lacZ* could be achieved by baculovirus transduction and that the expression levels were higher when the inoculum was replaced after 4 hours with fresh 20% MEM; b) that the efficiency of expression of the reporter gene was dose-related, ranging from 100% at $\frac{1}{2}$ and $\frac{1}{4}$ dilutions to < 1% at dilution 1/128; and c) that the baculovirus might have a toxic effect on the cells since the cell density in each well increased with the baculovirus dilution. An example of *lacZ* expression on EDC transduced with Bac-CMV / *lacZ* at a dilution of $\frac{1}{4}$ is illustrated in Figure 5.4.

Since the *lac Z* gene was cloned at the polyhedrin locus it is possible that during propagation of the virus in insect cells the recombinant gene had been expressed and the enzyme incorporated into virions. To determine whether the *lac Z* activity observed in the EDC transduced with Bac-CMV / *lac Z* was due to *de novo* synthesis of the enzyme and not as a result of the delivery of preformed enzyme into the cells, expression of the reporter enzyme was tested in the presence or absence of cycloheximide, a strong protein synthesis inhibitor. EDC monolayers were prepared in 96 well plates and grown overnight at 37°C and

inoculated with Bac-CMV / lacZ suspensions with or without cycloheximide at a concentration of 50 µg / ml. After a 4 hour incubation period at 37°C all the cells were washed with PBS and half of the wells were fixed with glutaraldehyde and stained with X-gal. The other half were incubated for a further 24 hours in the same medium. Then the cells were washed in PBS, fixed in glutaraldehyde and incubated with X-gal. Enzymatic activity was only detected in the 24 hour cultures that were incubated in the absence of cycloheximide (data not shown). These results indicated that the enzyme activity following baculovirus transduction was a result of *de novo* synthesis of β-galactosidase.

5.3.2.2. Sub-cloning of the EAVORF7 mammalian expression cassette from pcDNA3 / ORF7 into the baculovirus transfer vector pVL1393.

For expression of EAV ORF7 in EDC by baculovirus transduction it was necessary first to insert this gene into an appropriate baculovirus transfer vector for eventual recombination with linearised baculovirus DNA after cotransfection in Sf9 insect cells. Regulatory elements necessary for expression of heterologous genes in mammalian cells need to be present in the final recombinant baculovirus. Since these are not provided by the shuttle vector pVL1393, the cloning strategy, outlined in Fig. 5.5., was based on the insertion of the *NruI* – *SmaI* fragment of pcDNA3 / ORF7 into the multiple cloning site of pVL1393. This DNA fragment contains the EAV ORF7 gene downstream of CMV / T7 promoter sequences and upstream of the BGH polyadenylation site (poly A'). The restriction enzymes *NruI* and *SmaI* were selected for digestion of pcDNA3 since they are unique in the vector and are not present in EAVORF7. In addition, they generate blunt-end DNA fragments to which single adenosine residues

could be added at the 3'- ends of the molecule facilitating the rapid insertion into thymidine tailed vectors. Approximately 10 µg of plasmid pcDNA3 / ORF7 were digested with *NruI* for 4 hours at 37°C. Once the enzyme was inactivated for 15 min at 65 °C, the digest was purified and a second restriction digestion was performed with *SmaI* at 25 °C overnight. After inactivating the enzyme for 15 min at 65°C, approximately 6 µg of the digest was run on a 0.7 % agarose gel. This procedure separated two DNA fragments of approximately 2217 and 3562 base pairs corresponding to the expected sizes of the *NruI* – *SmaI* digested pcDNA 3 /ORF7 plasmid. The smaller DNA fragment was purified from the gel and A' residues were added to 3' ends before storing at – 70C.

The plasmid pVL1393 was digested with *SmaI* at 25°C overnight to generate a linearised DNA fragment which was subsequently T' tailed. Initially, the PCR reaction conditions used to generate the T'tailed pVL1393 were the same as those used for adding A' residues to DNA fragments. However, subsequent ligation reactions of insert and vector and subsequent transformation of competent *E coli* DH10B cells produced very few bacterial clones, all of which contain the pVL1393 without the *NruI* – *SmaI* fragment insert. The failure was attributed to inefficient T'tailing of the vector. When the concentration of dTTP was increased 10 times to achieve a final concentration of 20 mM, ligation was successful and transformation of the ligation reaction produced recombinants containing the insert and the T' tailed vector. Indeed, PCR- screening of bacterial clones using EAVORF7 specific primers identified five EAVORF7 positive clones, 3a, 2d, 2c, 2a and 1a (Fig 5.6.). Double and single restriction digests of plasmid preparations from three of these positive clones (3a, 2a and 1a) with

*Bam*HI, *Sna*BI and *Bam*HI and *Sna*BI (Fig. 5.7.a) failed to indicate presence of the ORF7 cassette for clones 1a and 2a but produced for clone 3a DNA bands which correspond to a recombinant plasmid in which the EAVORF7 expression cassette is in reverse orientation respect to the polyhedrin promoter of pVL1393 (314, 333, 1065, 1099 and 8921 for the double digests, 1065 and 10660 for *Sna*BI and 333, 1099 and 10303 for *Bam*HI). PCR reactions using EAV ORF7 specific primers and EAVORF7 2510001 primer and the polyhedrin specific primer pVL1393(f) (5'-AAATGATAACCATCTCGC-3') of plasmid preparations from clone 3a, 2a, 2d and two previously untested clones 3b and 4b indicated that while EAVORF7 PCR products were obtained for all the clones, only 3a and 2d produced PCR products using the second pair of primers (Fig.5.7.b). These results confirmed that the EAVORF7 expression cassette was inserted in reverse orientation to the PH promoter in these plasmids. Another restriction digestion of clone 3a plasmid was performed using HindIII and XhoI enzymes (Fig. 5.7.c) corroborated the orientation of the insert. Sequencing of the recombinant plasmids 3a, 2d and 3b using polyhedrin specific primers pVL1393 (f) seq and pVL1393(r) confirmed the orientation of the inserts in 3a and 2b pVL1393 vectors were in reverse relative to the polyhedrin promoter whereas the one of 3b was in the same orientation and that the sequence across the junctions was correct for all three plasmids. Sequencing also revealed that all three plasmids presented a substitution of two guanines for two cytosines at positions 1686 and 1687 and a deletion of a cytosine residue in the position 1797 of the pcDNA3. These positions are located downstream of the EAVORF7 and polyadenylation site on the SV40 promoter and are therefore unlikely to affect expression. A large scale plasmid preparation from clone 3a was made and this

plasmid was denoted as pVL / pcD / ORF7. Expression of the nucleocapsid protein was achieved in RK-13 cells after cationic lipid plasmid transfection of this plasmid and co-infection with Vaccinia MVA / T7 (not shown).

The same cloning strategy was used to try to make recombinant baculovirus pVL1393 based shuttle vectors containing pcDNA3 expression cassettes encoding either ORF5 or ORF6 of EAV. The latter derived from *NruI* and *SmaI* digested pcDNA3 / ORF5 and pcDNA3 / ORF6 plasmids which were made and supplied by Dr McAleer at the AHT. Only one recombinant plasmid was obtained for ORF5 which was denoted as pVL /pcD /ORF5.

5.3.3. Generation of recombinant baculovirus

To obtain recombinant baculoviruses, Sf 9 insect cells were co-transfected with pVL / pCD / ORF7 and Bsu36I digested BacPak6 AcNPV virus (Clontech). This baculovirus contains two *Bsu36I* restriction sites positioned within ORF603 and within the essential ORF1629 gene. These sites flank the *lacZ* gene at the polyhedrin locus. Digestion of the viral DNA with Bsu36I linearises the genome and deletes parts of the above mentioned genes as well as the polyhedrin locus. This viral DNA is unable to replicate or produce virus particles unless it recombines with a transfer plasmid vector containing baculovirus ORF's 623 and 1629. The plasmid pVL / pCD / ORF7 provides these baculovirus sequences necessary for recombination as well as the EAVORF7 mammalian expression cassette.

SF9 insect cells were transfected with 100 ng of Bsu36I digested BacPak6 and 500 ng of pVL / pCD / ORF7 or pVL / pCD / ORF5 and incubated at 28°C for 4

days. After this period the tissue culture fluid from the co-transfections was harvested and used in a plaque assay to isolate recombinant baculovirus clones. Decimal dilutions of the co-transfection harvest and of a BacPak6 virus stock were used to inoculate each of a series of Sf9 cell monolayers prepared in 6 well flat bottomed plates and a plaque assay performed as indicated in materials and methods. Several plaques were picked and all corresponded to recombinant baculoviruses.

Identification of recombinant baculoviruses was done by PCR with EAVORF7 primers 251001 and 251002 using DNA extracted from baculovirus infected cells or from virus particles contained in tissue culture fluid. Fresh cell monolayers were infected with each plaque pick and incubated for 3 days. When the cpe developed the cells were harvested and pelleted and washed in PBS. After resuspending the cells in TE buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA) the cells were lysed by adding an equal volume of lysis buffer (50mM Tris-HCl, pH 8.0, 5% 2-mercaptoethanol, 0.4% w/v SDS, 10mM EDTA). Proteinase K to a final concentration of 25 µg / ml and RNase A to a final concentration of 2.5 µg / ml were added and incubated for 30 min at 37°C. Then total DNA was extracted twice with an equal volume of phenol: chloroform for 5 min. and finally precipitated by the addition of 3M CH₃-COONa and ethanol. After a final wash in 75% ethanol the precipitate was dried and resuspended in TE buffer.

Results from PCR indicated that EAVORF7 could be amplified from most of the samples derived from infected baculoviruses. Four clones were selected for baculovirus transduction. Confluent monolayers of EDC's prepared in a 96 well plate were used to inoculate Bac / CMVT7 / ORF7 baculovirus clones 1, 2, 3, 4. BacPak6, EAV and mock infected wells were used as controls. For each clone 4

virus dilutions in SFMEM were used: undiluted, 2/3, 1/3 and 1/6. The cells were washed in SFMEM medium and then were inoculated with 100 µl of either EAVLP3A+ at an moi of 3, MVAT7 at an m.o.i. of 1 or SFMEM and incubated for 1 hour at 37°C. The vaccinia MVA/T7 and mock infected cells were washed again with SFMEM and were inoculated with 100 µl of the baculovirus suspensions and incubated for 4 hours at 37°C. All the wells were washed in SFMEM incubated in 10%MEM for 20 hours. The cells were then washed in PBS and fixed in 4% formaldehyde, 0.4% Triton-X-100 and subjected to immunofluorescence with the anti-N specific antibody SP07 and FITC conjugated anti-rabbit IgG. Results showed expression of the nucleocapsid antigen in EAVLP3A+ infected cells as well as in cells infected with MVAT7 and transduced with the recombinant baculoviruses (Fig. 5.8). Expression was not detected either in mock or BacPak6 infected cells or in cells inoculated with the baculoviruses alone. The results showed that all clones tested contained the CMVT7 / ORF7 expression cassette in their genome and that this cassette is functional in EDC's upon baculovirus transduction although MVAT7 co-infection was necessary for detectable expression. The N antigen localised mainly in the perinuclear region and in the nucleus of baculovirus transduced cells contrasting with EAVLP3A+ infected cells which show a widespread staining pattern of the cytoplasm with less proportion of cells stained in the nucleus (Fig. 4.1, Chapter 4). The efficiency of expression in baculovirus transduced EDC's was higher than 50% and indicated that baculovirus transduction could be applied in the CTL assay to attempt to determine proteins that hold CTL epitopes.

5.3.4. Attempts to measure EAV specific CTL activity using N expressing EDC targets.

An EAV CTL assay experiment was designed to test whether the EAV N expressing EDC's could function as targets for EAV cytotoxic effector cells. Transduction of EDC with the recombinant baculovirus Bac / CMVT7 / ORF7 was used to generate CTL targets expressing the N protein of EAV. Effectors, prepared as described previously, were obtained from pony 027a 10 months after infection with EAV. EDC from pony 027a were resuspended in 20% MEM medium at 4×10^5 cells / ml and 4 mls aliquots prepared. Each aliquot was inoculated with either EAV at an moi of 0.3, MVAT7 at an moi of 1, a combination of MVAT7 at an moi of 1 and 0.5 mls of Bac / CMVT7 / ORF7 or 0.5 mls of Bac / CMVT7 / ORF7 only. One aliquot remained uninfected. These cell suspensions were radiolabelled with 15 μ Ci / ml of ^{51}Cr and seeded onto two 96 well plates and incubated at 37°C for 24 hours. Effectors, a lysis solution or CTL medium were incubated for 4 hours with the targets of both plates and radioactivity of the medium measured as an indication of cell lysis. Specific lysis percentages were calculated as indicated in Chapter 4.

The results of this assay (Table 5.9) showed very little lytic activity of mock induced effectors against all the targets. In contrast, EAV induced effectors displayed different degrees of cytolysis on different targets. Thus, baculovirus transduced EDC co-infected with MVAT7 and EAV infected EDC were effectively lysed by the CTL effectors in contrast to mock infected EDC's which showed low percentage lysis values. The values obtained for EDC infected with MVAT7 indicated that EAV induced effectors exert some cytolytic activity on

these cells, however the results are not conclusive since there is a small variation in the percentage lysis figures between the two plates. The percentage lysis values for baculovirus transduced EDC targets varied substantially between both plates making the interpretation of the results difficult. This variation is caused by the difference in the spontaneous target lysis between the two assays. In summary, in this experiment it has not been possible to determine whether the nucleocapsid protein of EAV is an important antigen for EAV specific cytotoxic lymphocytes. Interpretation of the results has been complicated by the variability between replicates and by the low total ^{51}Cr release of targets inoculated with baculovirus (approximately half of the total release from EAV, MVAT7 or mock infected targets) which indicate that EDC either did not uptake or did not retain the radiolabel.

5.4. Discussion

The purpose of this work was to establish a method to identify EAV proteins that serve as CTL targets. The strategy employed used non-replicative vectors for the delivery of EAV genes into equine dermal cells for the expression and presentation of derived peptides by MHC-I which could be then recognised by CTL effector cells using the newly established methods to measure CTL responses against EAV.

We chose the nucleocapsid N protein as a potential CTL target since this protein is expressed abundantly in EAV infected cells and internal antigens in other virus systems have been shown to be frequently recognised by cytotoxic lymphocytes. Such antigens have been shown to contribute to the development of CTL

protective immune responses (Bangham *et al.*, 1986; McMichael *et al.*, 1986; Townsend *et al.*, 1984). Furthermore, the nucleocapsid protein N of LDV has been identified as a CTL target (Even *et al.*, 1995). We cloned successfully the nucleocapsid gene of EAV into pcDNA3 and showed that the recombinant gene was functional since the protein was detected by immunofluorescence in pcDNA3 / ORF7 transfected COS7 cells and RK-13 cells. However, expression was only detected when the cells were co-infected with recombinant vaccinia MVA / T7. This virus encodes the RNA polymerase of the phage T7 and hence enables transcription of the ORF7 gene of EAV from the cytoplasm. The lack of detectable expression in the absence of cytoplasmic transcription may result from the plasmid failing to reach the nucleus where the mammalian RNA polymerase II would have generated the ORF7 m-RNA. However, we have shown that transfection alone was sufficient to drive the expression of the lac Z gene in COS-7 cells from pcDNA3 / lacZ indicating that delivery of the gene to the nucleus occurred efficiently. This suggests that blocking of the expression of EAVORF 7 in the absence of MVA/T7 was gene specific rather than related to the plasmid. For example, the EAVORF7 may contain an early transcription termination signal or m-RNA splicing signals disrupting expression of full length m-RNA in the nucleus. Alternatively, the resulting m-RNA may be relatively unstable or poorly exported from the nucleus.

Although the EAVORF7 gene was expressed abundantly in COS-7 and RK-13 cells following transfection of pcDNA3 / ORF7, the efficiency of expression in EDC's was very poor making this approach not suitable for identifying CTL targets. All the plasmid transfection experiments were conducted using the non-

liposome lipid reagent FuGENE and it is uncertain whether other transfection techniques (use of cationic liposomes, electroporation, dextran, calcium phosphate) would have been successful for the efficient expression of EAVORF7. However, at the time these experiments were conducted, the potential of baculovirus transduction as a gene delivery system in equine cells was investigated in our laboratory. Heterologous genes have been expressed successfully using this technique (Condreay *et al.*, 1999). Similarly, *lacZ* gene expression was detected in equine embryonic lung cells transduced with a recombinant baculovirus encoding this gene under the CMV / IE promoter. This recombinant baculovirus also transduced and induced *lacZ* gene expression in EDC's., the cells used as targets in the EAV CTL assay. Therefore, the generation of a recombinant baculovirus containing the EAVORF7 under the control of the CMV promoter was pursued. The baculovirus bac / ORF7 was made by subcloning a *NruI-SmaI* fragment from the pcDNA3 / ORF7 into the *BamHI* multiple cloning site of pVL1393. This strategy was similar to that one used by Condreay *et al.*, (1999). However in the latter case the fragment subcloned was slightly longer to include the SV40 promoter-neomycin-phosphotransferase expression cassette necessary for generation of stably transfected cell lines. Baculovirus transduction of EDC's resulted in the expression of the recombinant gene in approximately 50% of the cell sheet, although again co-infection of MVA/T7 vaccinia virus was required, which reinforces the hypothesis that the blocking of the expression in its absence was gene related. The N protein localised mainly to the nuclei and to discrete perinuclear foci in transduced cells as opposed to EAV infected fibroblasts in which N antigen shows a more widespread distribution. Importantly, the cells

retained their original morphology indicating that the procedure was not highly toxic to the cells which is consistent with previous observations that indicate the low levels of toxicity of baculovirus transduction (Shoji *et al.*, 1997).

Baculovirus transduction was employed to assess the cytotoxic effect of EAV and Mock induced effector lymphocytes on Bac/ pcD / ORF7 transduced EDC's. The results of this experiment were not conclusive and further optimisation of the use of baculovirus transduction is required to obtain an answer to the question of whether this technique can be used to identify EAV CTL targets. Although as we discussed earlier, the EDC's did not lose their normal cell architecture, the total ⁵¹Cr release of baculovirus transduced targets was lower than that of EAV or mock infected EDC's suggesting an alteration in the uptake or retention of the radiolabel occurred during the overnight incubation period. This, in combination with the variability in the specific lysis between replicates, makes the assay difficult to interpret. It is important to note that baculovirus suspension was not removed after inoculation and this might have contributed to the observed decrease in the total release values of the baculovirus targets in comparison to the EAV, MVA/T7 or Mock targets. Further improvements of the current protocol should take this fact into account and test the effect of removing the baculovirus inoculum after a relatively short adsorption period.

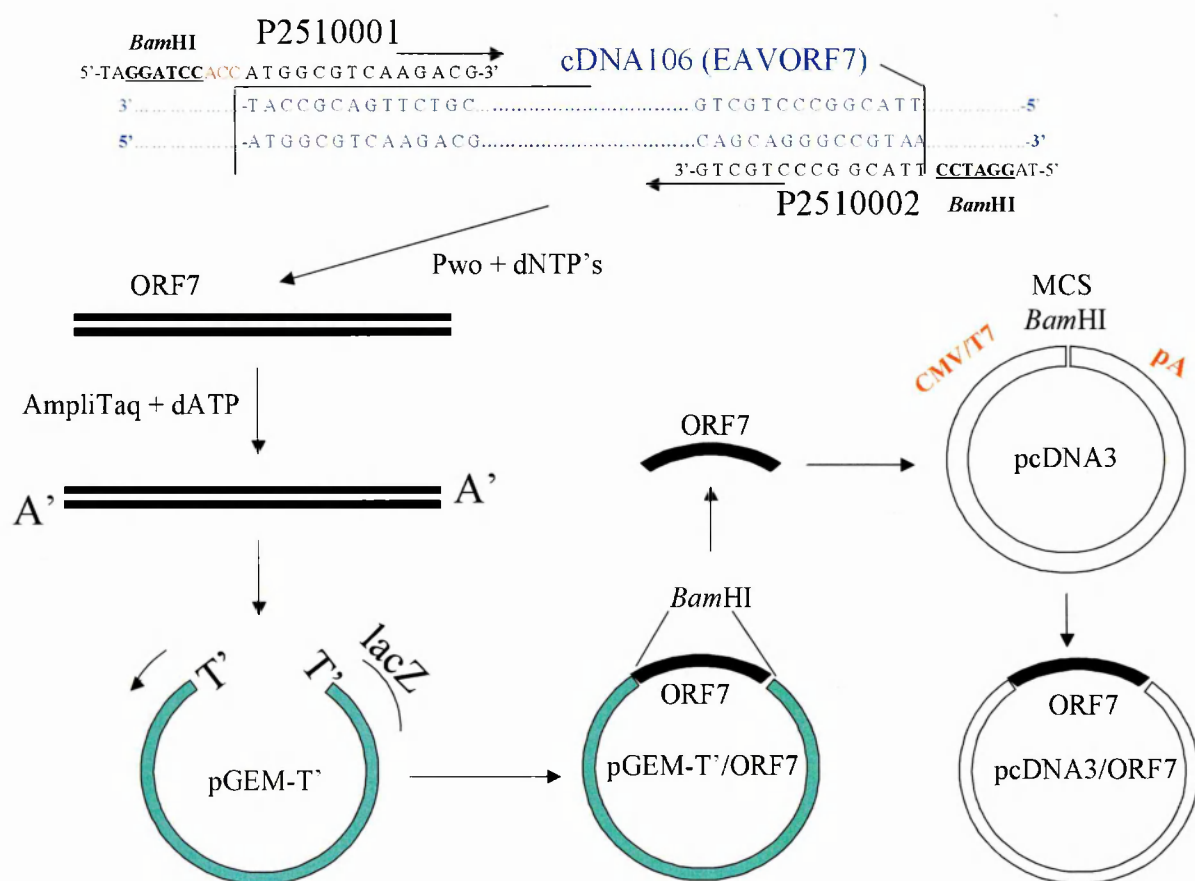


Figure 5.1. Cloning strategy to obtain an EAVORF7 mammalian expression plasmid vector. The cDNA clone 106 was used as a template to generate a PCR product using primers 2510001 and 2510002 as indicated in the text. BamHI restriction sites are indicated in bold and the additional nucleotides included in the forward primer to obtain a Kozak sequence are indicated in red. The PCR product was A' tailed and ligated into pGEM-T' as an intermediate step before digestion of pGEM-T'/ORF7 with BamHI and ligation of the digested ORF7 PCR product into the multiple cloning site of the mammalian expression plasmid pcDNA3.

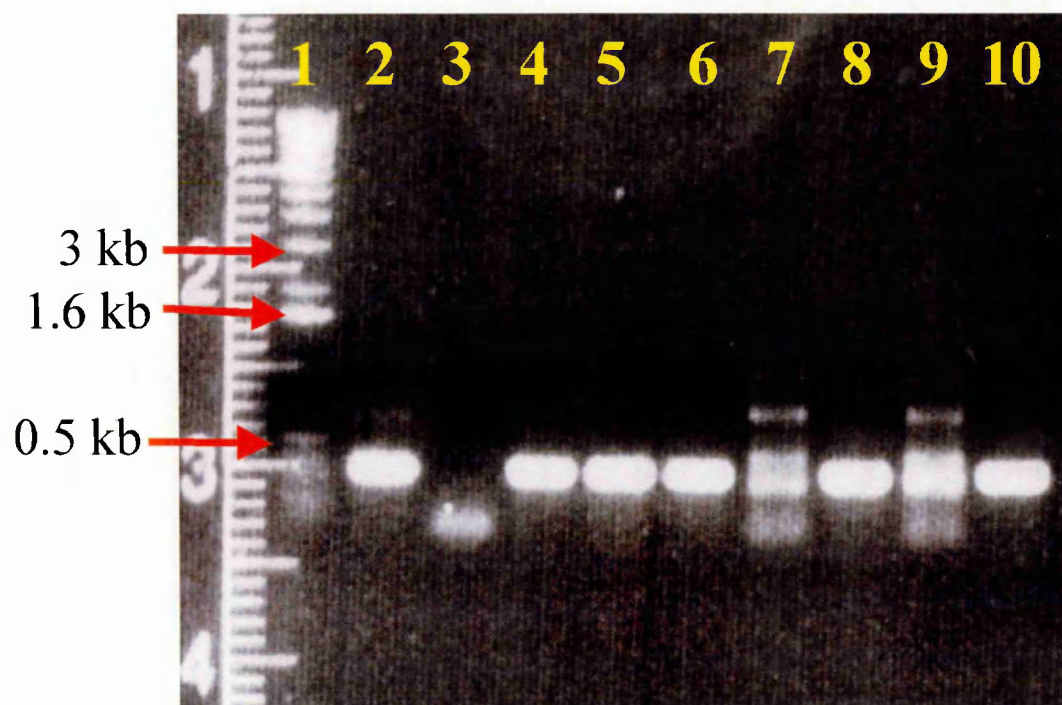


Figure 5.2. Sizes of DNA fragments from PCR reactions using primers EAVORF7 specific primers 250001 and 250002 on lysates of bacteria transformed with pcDNA3 and EVAORF7 ligation reactions. Lane 1 corresponds to the molecular weight marker 1 kb ladder, lane 2 corresponds to products from a positive control (pGEM-T/ORF7), lane 3 corresponds to a negative control (plasmid pcDNA3) and lanes 4-10 correspond to bacterial clones 3c, 1b, 1a, 1d, 1c, 3b and 4c.

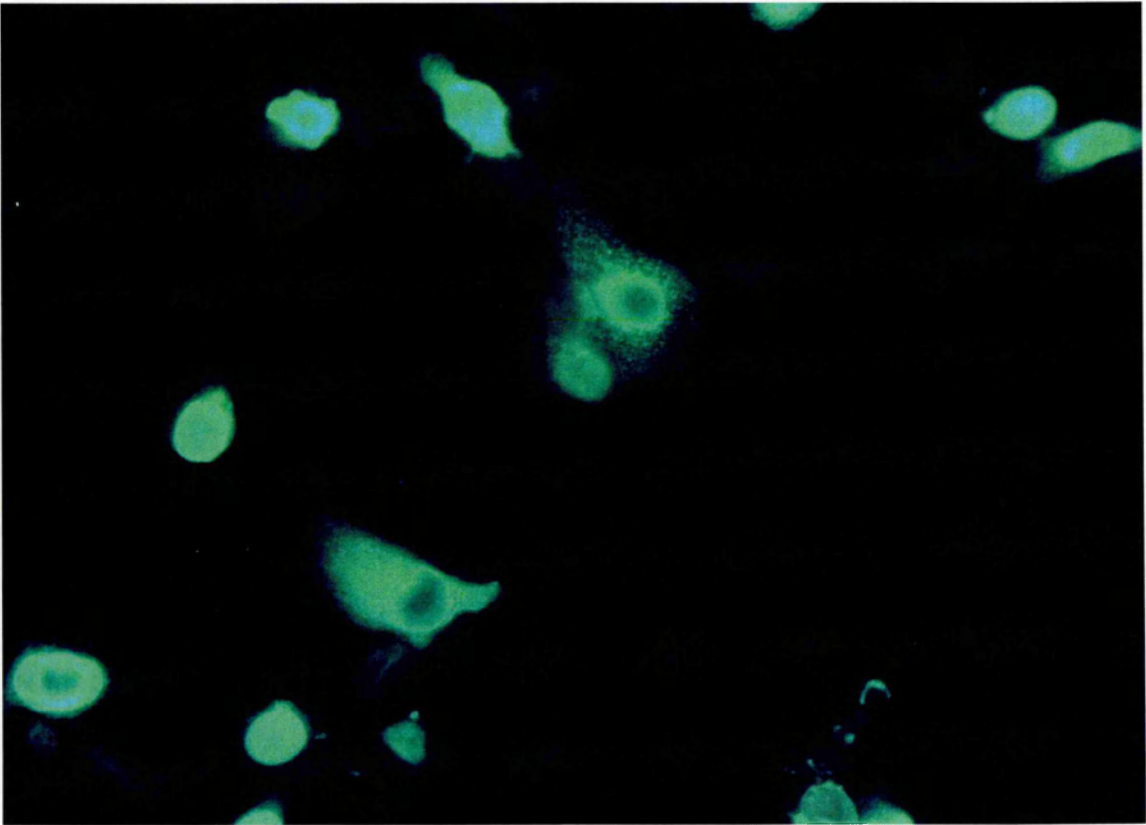


Figure 5.3. Expression of N antigen of EAV in COS7 cells transfected with pcDNA3 / ORF7 and co-infected with recombinant vaccinia virus MVA / T7. Detection of N was done by immunofluorescence using anti-SP07 rabbit polyclonal antibody of cells fixed at 24 hours post-infection.

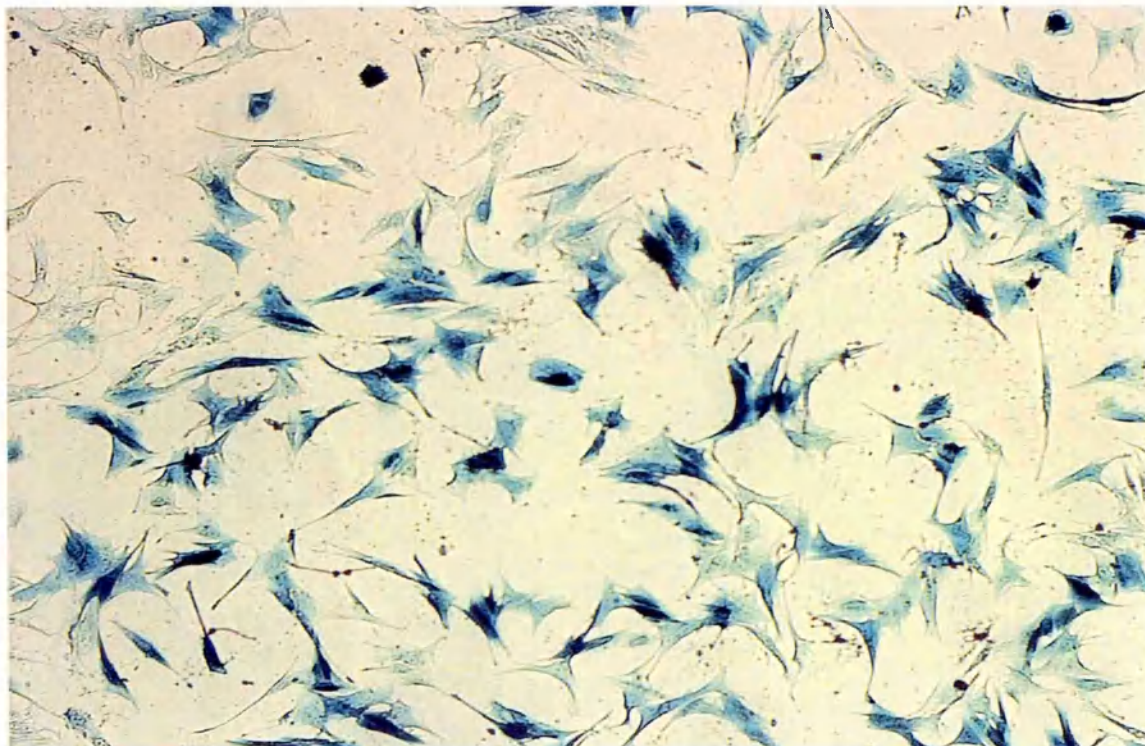


Figure 5.4. Expression of the lacZ gene in EDC transduced with recombinant baculovirus Bac-CMV/lacZ. EDC's were inoculated with the baculovirus preparation diluted 1:4 in 20%MEM, incubated for 24 hours at 37°C and stained with X-gal solution after fixation with glutaraldehyde.

Figure 5.5. Outline of the cloning strategy used to generate a baculovirus ‘shuttle’ vector for expression of EAVORF7 in EDC’s. The plasmid pcDNA3 / ORF7 was digested with NruI and SmaI restriction enzymes generating a DNA fragment with blunt ends containing the EAVORF7 expression cassette with the CMV/T7 promoter and polyadenylation signal (pA). This DNA was A’ tailed and inserted into a pVL1393 baculovirus shuttle vector previously digested with SmaI at the multiple cloning site, positioned immediately downstream of the polyhedrin promoter (PH), and treated to add thymidine (T’) to its 3’ ends. The new ‘shuttle’ vector pVL / pcD / EAVORF7 used to generate the recombinant baculovirus, which contains the EAVORF7 expression cassette in reverse orientation respect to the polyhedrin promoter, is represented in the figure. The relative positions of the restriction enzymes used in the characterisation of the recombinant plasmid and the theoretical sizes of the fragments they generate are shown. The black or blue colours indicate which enzymes have been used in the same restriction digestion experiment to characterise the recombinant plasmid. Green arrows represent primers 2510001 and pVL(f).

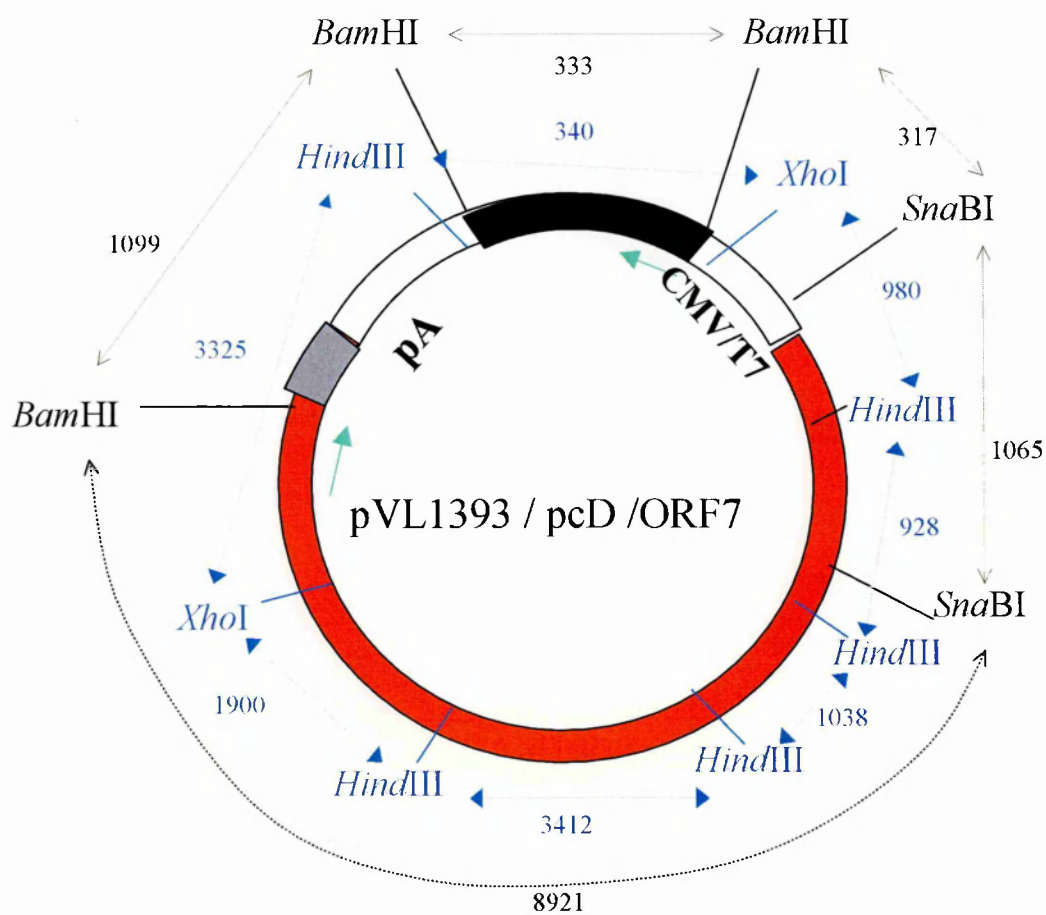
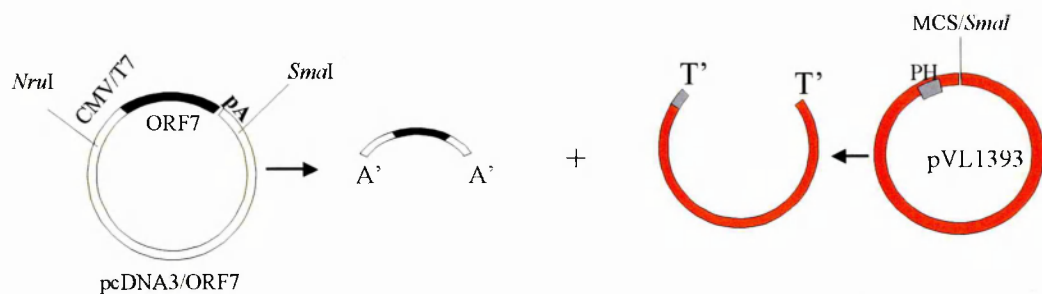


Figure 5.5.

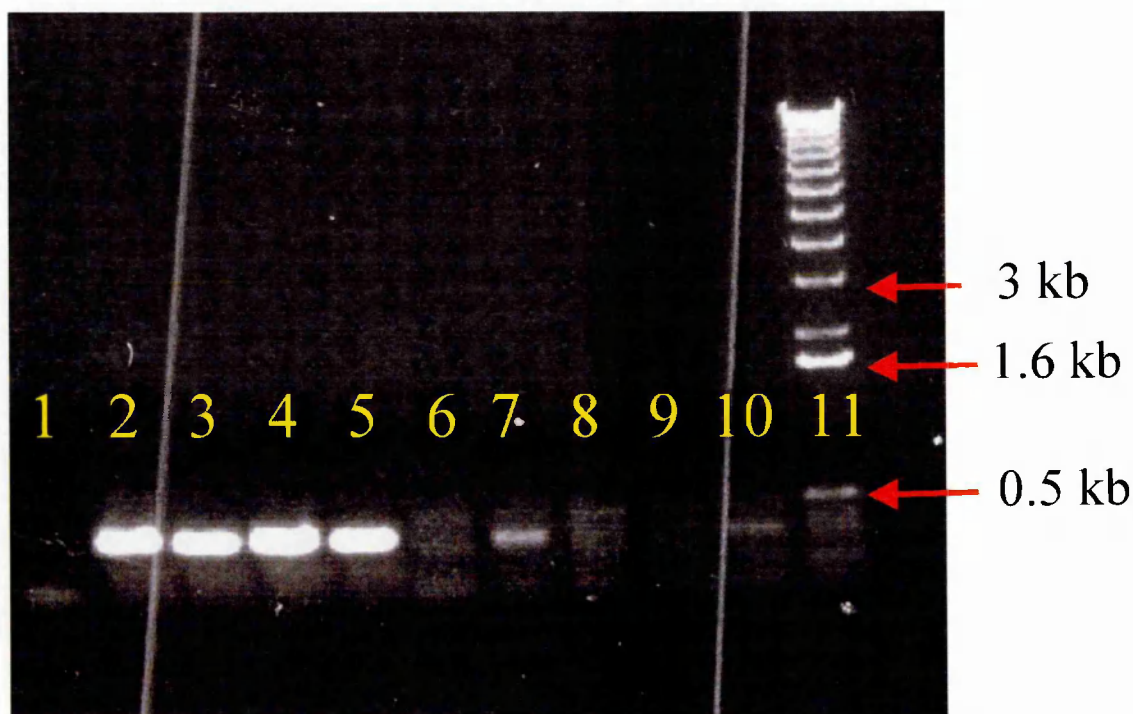


Figure 5.6. Sizes of DNA fragments from PCR reactions using primers EAVORF7 specific primers 250001 and 250002 on lysates of bacteria transformed with pVL1393 and pcDNA3 / ORF7 ligation reactions. Lane 1 corresponds to products from a negative control (plasmid pcDNA3), lane 2 corresponds to a positive control, (pGEM-T/ORF7), lanes 3-10 correspond to bacterial clones 3a, 2d, 2c, 2b, 2a, 1d, 1c and 1a and lane 11 corresponds to the molecular weight marker 1 kb ladder

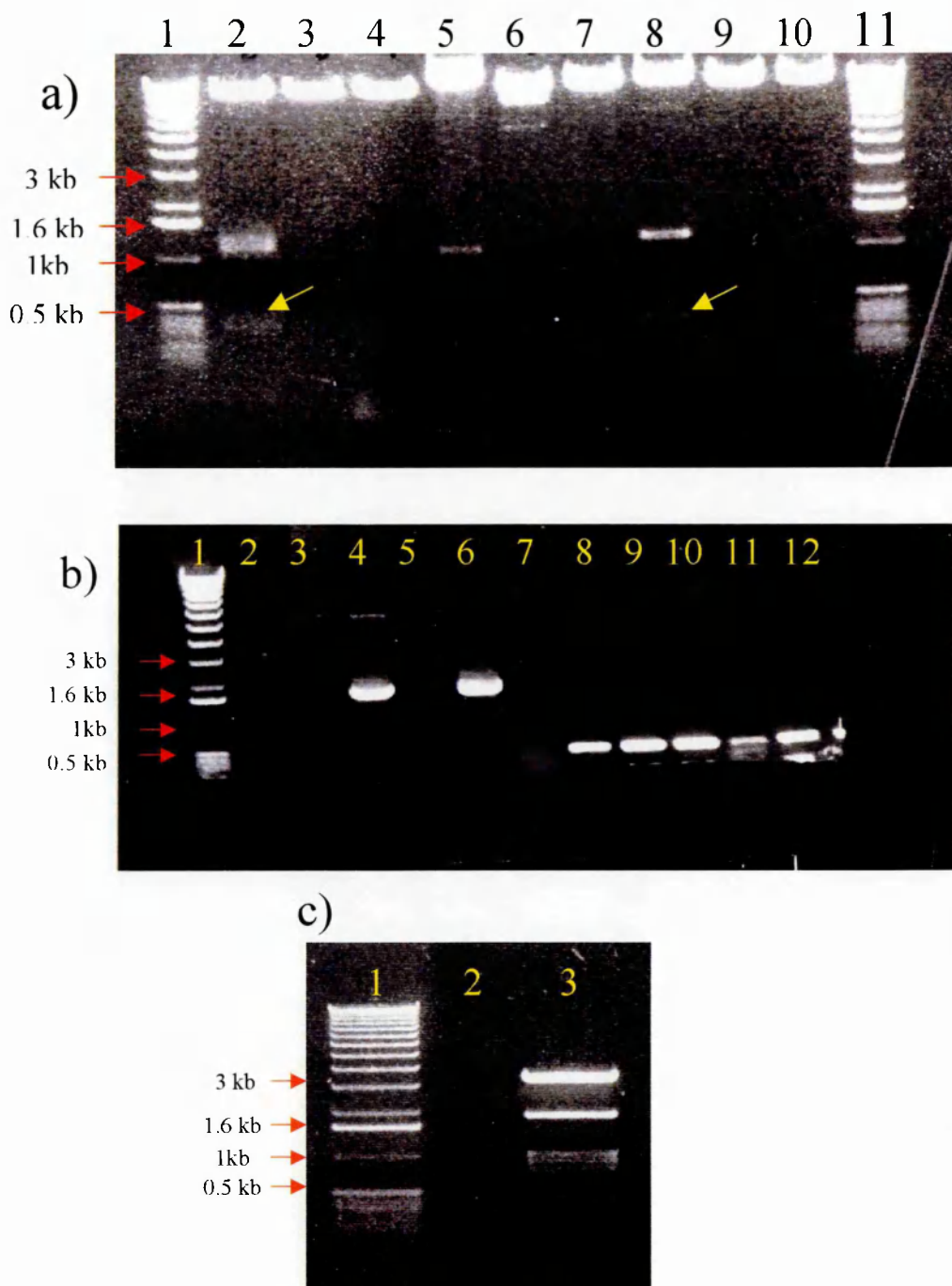


Figure 5.7. Selection of pVL1393 / pcD / ORF7 positive bacterial clones. A) Sizes of DNA fragments from *Bam*HI / *Sna*BI double restriction digests (lanes 2-4), *Sna*BI restriction digests (lanes 5-7) and *Bam*HI digests (lanes 8-10) of plasmid preparations from clones 3a (lanes 2, 5 and 8), 2a (lanes 3, 6 and 9) and 1a (lanes 4, 7 and 10). Lanes 1 and 11 contain 1kb DNA ladder. Faint ORF7 DNA bands fragments are indicated by yellow arrows. B) PCR reactions using primers EAVORF7 specific primers 250001 and 250002 (lanes 8-12) or primer 250001 and pVL (f) (lanes 2-6) on plasmid preparations from clones 3a (6, 12), 2a(5, 11), 2d (4, 10), 3b (3, 9) and 4b (2, 8). Lane 1 contains the 1kb molecular weight marker. Lane 7 is empty. C) Sizes of DNA fragments from *Xho*I and *Hind*III double restriction digests (lane 3) of plasmid 3a. Lane 1 contains the 1 kb molecular weight marker. Lane 2 is empty.

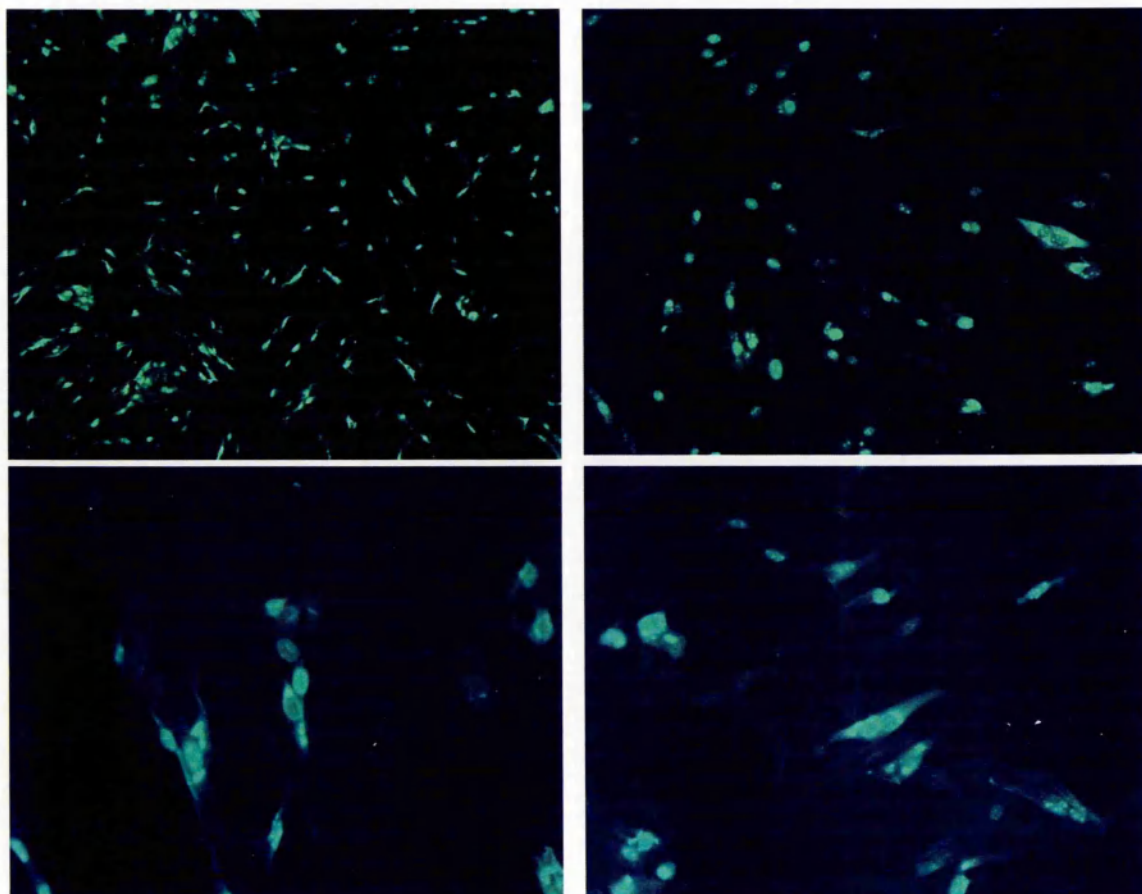


Figure 5.8. Expression of EAV N protein in EDC's transduced with the recombinant baculovirus pVL / pcD / ORF7 and co-infected with recombinant vaccinia MVA / T7. Expression was detected by IFI using α -SP07 rabbit polyclonal antibody 24 hours after transduction. Pictures were taken at 100x (a), 200x (b) and 400x (c and d).

Effectors	EAV	MVAT7	MVAT7+BacORF7	BacORF7	Mock
Mock stimulated	4998.66 6287.925 6147.37 4.361633 6882.68 7122.99	4203.95 5244.518 4848.66 3.791831 5813.67 6111.79	3841.22 4213.983 3409.67 2.61466 5200.47 4404.57	4272.96 4157.628 4126.05 6.068562 3784.94 4446.56	4642.36 4060.62 4850.73 -4.649625 3274.37 3475.02
EAV stimulated	8754.67 9947.05 10083.76 25.01016 9673.21 11276.56	7060.3 6990.945 7864.16 14.72819 6726.38 6312.94	6081.26 5677.563 7671.23 21.56068 4745.22 4212.54	5899.21 5870.275 6014.86 27.78338 5171.09 5705.94	6039.41 6309.198 6957.16 6.236734 6038.77 6201.45
Tx100 Total release	24568.04 23236.23 25251.29 20219.56 22906.01	20185.64 20608.11 23084.89 19115.2 20046.7	12165.69 11737.89 11204.88 11295.94 12285.05	11144.23 11566.98 11808.26 11149.12 12166.32	25095.71 25676.95 22988.38 27834.03 26789.69
Spontaneous release	5280.98 5515.99 5177.49 6658.4 4947.09	4868.63 4639.205 3848.19 5263.24 4576.76	3908.07 4012.075 4421.15 4376.34 3342.74	3407.58 3679.945 3634.96 3409.77 4267.47	3899.18 5021.683 6618.24 5758.39 3810.92

Effectors	EAV	MVAT7	MVAT7+BacORF7	BacORF7	Mock
Mock stimulated	5816.87 5096.238 4636.37 4.75429 4808.32 5123.39	4544.7 4799.553 3943.11 -8.525275 3599.23 4711.17	4680.14 4533.175 4759.95 1.151178 4680.56 4012.05	3971.1 4533.45 4552.73 -14.02905 5733.45 3876.52	5464.65 5721.955 6084.94 -0.656345 4906.38 6431.85
EAV stimulated	11340.15 10558.3 10538.13 30.43392 10391.14 9963.76	7538.12 6688.003 6042.94 7.468362 5883.18 7287.77	5739.05 5400.243 4279.74 14.08476 5724.27 5857.91	4474.7 5018.283 4407.98 -6.656288 5390.28 5800.17	4950.22 6439.12 6213.88 3.169147 6517.77 8074.61
Tx100 Total release	26052.75 26075.24 25999.38 24786.61 27462.2	21501.66 21085.73 18726.1 21233.22 22881.93	11035.4 11160.21 10540.38 12441.83 10623.23	11499.86 12032.25 12724.19 11754.89 12150.04	24680.02 24592.18 27205.81 23509.48 22973.41
Spontaneous release	4008.16 4805.465 3879.38 4870.63 6463.69	4795.86 5526.875 5573.75 6489.15 5248.5	4616.02 4456.313 4682.47 3910.76 4616	5057.12 5456.925 6804.87 4547.12 5418.59	5692.23 5845.928 6185.08 5017.39 6489.01

Table 5.9. EAV specific cytotoxicity measured against EAV N expressing targets. The cytotoxic effect of 027a effectors was evaluated against four replicates of five different target cells. The 51Cr release of each replicate is expressed in cpm and the mean was calculated. This mean value (*in italics*) was then used to calculate the percentage of specific lysis for each set of targets (*in bold*). The experiment was performed in duplicate.

Chapter 6: General discussion

In this project, two novel strategies for the generation of marker vaccines against EAV have been investigated and new methods have been developed to analyse the immune response of the horse to vaccination and infection with this virus. New knowledge has been gained on the understanding of adaptive immune responses that operate in clearance of EAV infection.

Marker vaccination is a concept applied in medical and veterinary sciences in which the induction of immune responses in the individual are both protective against the particular disease/infection under investigation and also, can be differentiated from those elicited by natural exposure to the pathogen. This vaccination strategy allows prevention of diseases and / or infections in immunologically naïve and susceptible animals and differentiation of these from naturally infected (and potentially infectious) animals. This in turn permits the simultaneous application of disease eradication and vaccination policies, which may otherwise be incompatible, as is the case for foot-and-mouth disease and classical swine fever in the European Union. The advent of marker vaccination is particularly advantageous for the control of virus diseases in which a persistent infectious carrier state exists, such as foot-and-mouth disease, or in those which induce latency (BHV-1 in cattle, Aujeszky's disease of pigs or EHV-1 in horses) and have the potential to initiate an outbreak upon reactivation. In the case of BHV-1 and Aujeszky's disease, eradication policies have been applied in several European countries and marker vaccine immunisation forms an important part of those programmes (van Oirschot, 2001). If the vaccine is a key player in these

control programmes, of no less importance is the application of serodiagnostic tests capable of detecting the differential antibody responses in vaccinated and naturally infected animals. The huge development of recombinant DNA technology in the past two decades has made possible the development of marker vaccination strategies against many viral infections of domestic animals (van Gennip *et al.*, 2002 ; Pensaert *et al.*, 1990; Bouma *et al.*, 2000; van Oirschot, 2001, Babiuk, 1999).

EAV causes a systemic infection in horses, which may result in abortion or neonatal foal death and the establishment of persistent infections in stallions. The latter has the potential to facilitate transmission of infection via the venereal route. Although the infection frequently passes clinically unnoticed, its abortigenic potential, the capacity to persist in stallions and the resultant restrictions imposed on trade, make this disease economically important. EAV is has a worldwide distribution but its prevalence varies significantly between geographical regions. This is the case in central European countries (areas of suspected high prevalence) and the UK (very low prevalence). The constant movement of horses between these two regions of different prevalence poses a risk of introduction of the disease to naïve populations via persistently infected stallions or their semen. The control of EAV in the regions of low prevalence such as the UK, relies heavily on prophylactic sanitary measures based on accurate and thorough serological surveillance of the breeding stock. The use of a whole virus inactivated vaccine in stallions in some countries complicates serodiagnostic interpretation. Hence, knowledge of vaccination history is critical to accurate diagnosis and relies on co-operation between diagnostic laboratories,

veterinary surgeons and horse owners/trainers. In this situation, the use of an effective marker vaccine should not only provide protection against the disease should this be introduced and reduce aerosol virus transmission, but it should not compromise sero-surveillance.

The studies presented in this thesis evaluated the potential of two different marker vaccination approaches for EAV focusing in the analysis of the immune responses elicited and their protective efficacy against virulent challenge. The strategies employed were quite different, one based on a single purified EAV antigen (the 6hisG_Lecto) administered intramuscularly with adjuvant and the other based on a recombinant live virus lacking an immunodominant region of G_L and administered intranasally. It was therefore not surprising that fundamental differences were observed with regards to the type of immune responses that developed and protective efficacy. These vaccination studies not only have value for their application in prevention of EAV infection, but serve as tools to dissect and study the development of adaptive immune responses against virus infections in the horse. Furthermore, these studies serve as a vaccinology model which can be applied to the study of other related viruses that infect horses or other domestic animals, such as PRRSV of pigs.

6.1. EAVG_L as a sub-unit vaccine.

The potential for EAVG_L as a sub-unit vaccine for EAV was based on the findings of Chirnside *et al.* (1995a) who demonstrated that immunisation of ponies with an immunodominant region of G_L resulted in the induction of VNAb responses. By using the recombinant protein 6hisG_Lecto (comprising all domains contributing to the formation of VNAb epitopes) as the sole immunogen in

combination with the Carbopol adjuvant, the VNAb response in ponies was improved over that reported by Chirnside *et al* (1995a). This response was protective against challenge with the LP3A virus strain and the degree of protection correlated with serum VNAb titres at the time of challenge. As expected for an inactivated vaccine, protection from infection was dependent upon the amount of serum antibody and was not complete when VNAb titres declined to low levels. In particular, the amount and duration of viraemia was not reduced in animals with low VNAb titres compared with unvaccinated controls. However, even in animals with low VNAb, reduction of virus respiratory excretion was still evident which would reduce the spread of infection in an outbreak situation.

The findings presented in Chapter 2 were corroborated in follow up experiments performed with commercial support (Boehringer Ingelheim VetMedica). In one study, 4 groups of 8 ponies were vaccinated with Carbopol adjuvanted vaccines containing either no antigen (placebo control), 4 µg, 8 µg or 32 µg of the 6hisG_Lecto protein. The vaccines were administered by the intramuscular route in two doses, 4 weeks apart. VNAb responses were measured and protective efficacy against challenge with EAVLP3A⁺ in the same way as described in Chapters 2 and 3. All vaccinated ponies responded with a VNAb response peaking 2 weeks after the second dose was administered (mean VNAb titres 2.5) with very little differences between vaccinated groups (Fig. 6.1.). By day 112, VNAb were no longer detectable and remained at this level until the ponies were challenged on day 211 (approximately 7 months). Vaccinated ponies mounted a strong anamnestic response to virus infection and VNAb titres peaked sooner in

these animals than in those that received the placebo. Consistent with the faster response to virus infection and with the findings reported in this thesis, vaccinated ponies showed partial protection against EAVLP3A+ infection despite having undetectable pre-challenge VNAb titres. Whereas the virus excretion from the upper respiratory tract was reduced, both in amount of virus excreted and in duration of virus shedding (Fig.6.2.b), reduction of duration of pyrexia (Fig.6.2.a) and viraemia (Fig. 6.2.c) were more subtle. The levels of protection achieved appeared to be sub-optimal. Unfortunately, comparison with whole virus vaccines could not be made because this parameter was not included in the study design. Furthermore, comparison with published work was not possible because in most of these studies, group sizes were very small or animals were challenged with virus shortly after vaccination. In the absence of comparative vaccination / challenge studies the relative protective efficacy and duration of immunity afforded by the inactivated vaccines versus 6hisG_L sub-unit vaccine is uncertain. The reduction of virus excretion with the potential prevention of respiratory spread provided by 6hisG_Lecto vaccination, which offers the additional advantage of differential diagnosis against natural infection, makes the sub-unit vaccination approach an interesting prophylaxis strategy for equine viral arteritis. In-contact transmission studies are necessary to determine the minimum nasal virus excretion levels required for aerosol spread of infection.

The kinetics of the serum VNAb responses following 6hisG_Lecto vaccination of ponies was not unexpected for a non-replicating anti-viral vaccine and similar results have been observed with other Carbopol-based vaccines in horses (Mumford *et al.*, 1988). It is possible however, that alternative vaccination

regimes and the use of other adjuvants may extend the duration of serum VNAb's. Re-vaccination 6 months after a primary 2-dose course of whole inactivated EAV virus vaccine prolonged the duration of VNAb for at least 6 months (Fukunaga *et al.*, 1990). The same effect was observed with a commercial whole virus inactivated vaccine in a field study which showed a correlation between the VNAb titres and vaccination history in stallions (Cardwell *et al.*, 2002). Prolonged antibody responses after inactivated vaccine administration have been observed also in ponies immunised with equine influenza ISCOM vaccines (Mumford *et al.*, 1994a).

6.2. EAV-GLA immunisation provides complete protection in the light of low titre VNAb response against wild type EAV.

A live marker vaccination strategy was also explored. Deletion of an immunodominant region of the envelope G_L protein had no apparent effect upon the ability of the vaccine virus to replicate *in vivo*. The vaccine induced an asymptomatic infection in ponies but provided full protection against a virulent challenge despite induction of a very weak VNAb response. This vaccination strategy offers a number of advantages compared with inactivated whole virus vaccines as summarised in Table 6.4. The main disadvantage is the requirement to demonstrate safety. Although ponies infected with EAVGLA did not show clinical signs, our studies did not demonstrate this feature was dependent on the deletion since we did not include a group vaccinated with the parental virus, i.e. a virus derived from cells transfected with the original cDNA clone. At the time of writing, a study comparing replication and immunogenicity of EAVGLA and the parental virus (designated EAV515) in ponies showed that there were no

differences in clinical signs, virus excretion or viraemia between ponies infected with either virus (Castillo-Olivares, unpublished observations). These results indicated that it is not possible to attribute the low virulence of EAVGL Δ to the deletion of 66-112 G_L peptide and therefore further studies are necessary to demonstrate that the deletion mutant virus does not revert to virulence after passage *in vivo*. However, studies in stallions demonstrated that unlike wild-type EAV viruses, the cDNA clone derived virus EAV030H was genetically stable and not prone to the generation of novel genetic variants of increased virulence (Balasuriya *et al.*, 1999b). It can be inferred therefore, that cDNA 030H derived viruses and those derived from its derivative plasmid pEAN515 share the same biological properties. Equally important from the safety point of view is to know whether the post-infection viraemia could result in abortion in the pregnant mares.

These concerns need to be addressed before using the EAVGL Δ as a marker vaccine but there are some methods that could be applied to improve safety. First of all, it would be possible to adapt the virus to grow in non-equine cell lines in the same way as the current modified live virus vaccine was generated and reduce its capacity to replicate *in vivo*. Alternatively, a 'knock out' deletion could be introduced in the virus genome which could be rescued by a complementing cell line supplying *in trans* the deleted gene. This virus carrying the knock-out gene would be able to infect cells *in vivo* but would be replication deficient. Interestingly, recombinant sub-unit (Hulst *et al.*, 1994; van Rijn *et al.*, 1996; Bouma *et al.*, 2000) and live virus marker vaccines (van Gennip *et al.*, 2000; 2002) have been developed for classical swine fever (CSFV) using similar

approaches to those described in this thesis for the prevention of EAV. The CSFV genetically modified live virus vaccines make use of a cDNA full-length clone of CSFV to replace the region encoding the E2 protein (the immunodominant antigen) with the E2 coding sequences of the related bovine viral diarrhoea virus (BVDV). Alternatively, an CSFV E2 expressing cell line was used to complement a CSFV E2 deficient genome to generate *in vivo* replication deficient virus. Both approaches provided protective immunity against CSFV infection. The replacement of E2 gene of CSFV by that of BVDV provided a positive serological marker. This is a strategy that has potential for EAV since replacement of the ectodomain of G_L by the equivalent region of PRRSV or LDV was shown to result in the generation of viable viruses capable of replicating in RK-13 and BHK-21 cell lines (Dobbe *et al.*, 2001). These studies suggested that EAVG_L ectodomain does not determine cell tropism and it is not essential for virus infectivity. The latter assumption is supported by our studies and extends this observation to *in vivo* infections. However, it must be recalled that the G_L ectodomain was only partially deleted in the present studies.

6.3. Structure of EAV VNAb epitopes - additional VNAb epitopes in other parts of the virion.

All known VNAb epitopes of EAV have been mapped to the ectodomain of G_L and most of the genetic variation results from changes in the immunodominant region of G_L which is most frequently recognised by field EAV VN positive sera. However the VNAb depletion experiments (Chapter 2) suggest that other VNAb epitopes may exist in addition to those represented by Peptide 2 (81-96) and 6hisG_Lecto (18-122) antigens. These could be conformational epitopes located in

G_L as has been demonstrated in various studies (Balasuriya *et al.*, 1995a, 1997; Deregt *et al.*, 1994) or linear and conformational epitopes present elsewhere in the virus envelope. The neutralisation activity of equine antiserum raised against wild type EAV and EAVG_LΔ (Chapter 3) for wild-type EAV and EAVG_LΔ does not support the hypothesis that important VNAb epitopes are present in other proteins apart from G_L. Otherwise, wild type EAV viruses would have been efficiently neutralised by ΔG_L- specific equine antiserum. However, new information on virus structure coming from very recent studies (Wieringa *et al.*, 2002; 2003) demonstrates that GP4 and GP3 are structural proteins and form heterotrimer complexes with GP2b (Gs), and that antibodies in EAV equine antiserum are directed to GP3 (Hedges *et al.*, 1999). Moreover, GP4 of PRRSV is known to contain VNAb epitopes (Meulenberg *et al.*, 1997) which indicates that additional neutralising epitopes of EAV might be formed by these three proteins.

Analysis of the antigenic specificity of EAVG_LΔ antiserum obtained during this project by western-blot or immunoprecipitation would provide useful information about the antigenic structure of EAV neutralising epitopes.

6.4. Detection of CD8+ T-lymphocyte cytotoxic precursors and their potential role in clearance of viral infection.

Many studies have associated virus neutralising antibodies with protection against EAV infection. This is supported by challenge studies in ponies vaccinated with inactivated whole virus or G_L sub-unit vaccines. However, evaluation of the protective effect of other important immunological effector systems was not possible previously because of the lack of methods to evaluate

them. The hypothesis that some of these adaptive responses may play an important role in EAV immunity is supported by the studies reported herein.

Table 6.3. Comparison of two different marker vaccination strategies for EAV.

EAVGLA	6HISGLECTO / CARBOPOL
Protective efficacy very high. Good prevention of viraemia – Potentially protective against abortion.	Protective efficacy high when VNAb titres are high. Less so when VNAb's are low.
Duration of immunity, at least 6 weeks. Potentially long lasting	Duration of immunity is short. Could be improved – adjuvants, dose regime.
Complete immune response: humoral, CTL's, mucosal immunity.	Humoral immunity. CTL immunity less likely. No mucosal immunity although could be induced if used mucosal route.
Heterologous protection. Higher potential to protect against different strains.	Heterologous protection. Lower potential to protect against different strains.
Differential diagnostic test available (Peptide-1 ELISA), developed and thoroughly tested.	Differential diagnostic test available (N-ELISA). Further development and validation required.
Safety. Unknown. Demonstration of safety requires extensive and expensive studies.	Safety. Potentially very safe. Safety studies easier to perform.
Stability and 'shelf life'. Dependence on cold chain. Lyophilisation could be a solution.	Chemically defined immunogen. Potentially very stable. Less dependent on cold chain.

Indeed, the fact that EAVG_LΔ infected animals were highly protected against infection in the absence of a strong VNAb response and the finding that EAV specific CTL responses could be detected in EAV-infected ponies suggested that cellular immunity could play an important role in protection against infection.

The contribution of CTL responses to clearance of EAV infection deserves further investigation and the deletion mutant virus would be a valuable tool for this. Comparing immune responses and immunity against virulent challenge of two groups of ponies vaccinated with live and inactivated EAVG_LΔ would provide further evidence of the contribution of CTLs to protection against infection. Furthermore, analysis of EAV-specific antibody responses in the respiratory mucosa, specifically measuring IgG, IgA and VNAb in nasopharyngeal washes would indicate whether mucosal immunity was likely to have contributed to the efficient immunity observed in EAVG_LΔ infected ponies. Indeed, it is becoming increasingly clear from other reports that the nature of induction site determines the type of immune response that is generated (Esser *et al.*, 2003). Thus, nasopharyngeal administration of EAVG_LΔ possibly induced strong secretory IgA and CTL responses at the site of virus entry and local generation of memory T cells (both effector memory and central memory T cells) and provided immediate protection with a rapid recall response. Continuation of the work described in this project would help to characterise the mechanisms of induction of efficient antiviral immunity in the horse and the factors influencing these responses.

6.5. Cellular immunity and persistence

During the course of EAV experimental infections conducted in the evaluation of 6hisG_Lecto vaccine, virus was isolated intermittently from the blood leucocytes of some control ponies between days 21 and 72 post-infection. Such long duration viraemias have been reported previously from EAV experimentally infected animals. For example Neu *et al.*, (1988) isolated EAV from blood cells of EAV infected stallions and showed that in 6 animals, the duration of viraemia lasted for 21, 26, 33, 40 58 and 111 days after infection. This study used entire males unlike the experiments reported herein. However, EAV has also been isolated from blood leucocytes in two of 12 castrated male horses at days 30 and 57 post-infection (McCollum *et al.*, 1986). The persistent carrier state of EAV has been described only in the sexually mature male, in which the virus replicates in tissues of the urogenital tract, particularly the *vas deferens* and *ampulla*. The maintenance of this state appears to be dependent upon the secretion of testosterone. However, this does not exclude the possibility that other sites of persistence exist and that the male reproductive tract is a secondary persistent site, which has been seeded by white blood cells permanently infected with EAV. The male reproductive tract is an immunologically 'privileged' site where immunosuppressive secretions (Anderson and Tarter, 1982) and predominance of CD8⁺ suppressor T-cells (Ritchie *et al.*, 1984) may impede clearance of virus infection by the immune system. However, if anatomical sites of persistence existed other than the male reproductive tract, there would be several other potential mechanisms for immune evasion by EAV. For example, there is the potential for generation of CTL escape mutants, induction of CTL apoptosis or modification of cytokine profiles of responding lymphocytes to reduce the

effectiveness of immune responses. Interestingly, PRRSV infections of pigs have been shown to persist for 3 or 4 months and they are not restricted to the male reproductive tract but involve lymphoid tissues of the oropharyngeal region. It is possible that after the onset of the VNAb response, EAV replication in the horse is restricted to specific cell types in lymphoid tissues until infection is finally cleared by CTLs. During this time, genetic variants with different biological properties might arise, a process already demonstrated to occur in the reproductive tract of stallions (Balasuriya *et al.*, 1999a), which could include the appearance of CTL escape mutants. The time required to finally control the infection would depend on various factors including capacity of the virus to infect tissues inaccessible to cells of the immune system and the genetic background of the host, in particular the capacity to present EAV peptides to CTL's (which depends on MHC-I. haplotype). The generation of CTL escape mutants is a mechanism employed by retroviruses such as equine infectious anemia virus (EIAV) and acquired immunodeficiency syndrome virus (AIDS) in humans (McGuire *et al.*, 2002; Borrow *et al.*, 1998).

The potential induction of apoptosis in bystander cells by EAV infection, already demonstrated in PRRSV infections *in vivo*, could result in protection from CTLs in infected monocytes-macrophages located in lymphoid tissues. Alternatively, expression of IL-10 could be upregulated in infected macrophages, as demonstrated in PRRSV infection of pigs (Suradhat *et al.*, 2003), resulting in inhibition of inflammatory responses and diversion of the immune response from a Th1- towards a Th2-type regulatory response with the resulting decrease of CD8+ T-lymphocyte effector functions, i.e. cytolysis and IFN- γ secretion.

The new development of methods to evaluate CTL responses will increase our capability to understand how EAV is finally cleared from the infected horse and to determine the factors affecting virus persistence. CTL responses of persistently-infected and non-persistently-infected stallions could be compared directly to determine whether there is a deficit in this type of immune responses in the former animals. Also, nucleotide sequence analysis of isolates collected from long-term viraemic ponies infected with the EAVLP3A⁺ strain, available from the EAV vaccine efficacy studies, could be used to determine the heterogeneity of the virus populations present in the samples collected. Of particular interest would be the identification of differences between these and the original inoculated virus and to determine the speed with which new mutations arose. Some of these mutations could cluster around the well-known VNAb epitopes or CTL epitopes. Identification of the latter was an objective of the present project.

6.6. Identification of viral proteins serving as targets for CTL's.

The objective of the last part of this project was the establishment of methods to identify proteins serving as targets for EAV specific CTLs. Target cells transiently expressing EAVORF7 were used in the standard CTL assay previously described (Chapter 4). Unlike plasmid transfection, baculovirus transduction appeared to be a suitable method to achieve protein expression in a high proportion of EDC. The levels of expression of EAV N antigen in transduced EDC, which occurred without the loss of their original morphology, suggested *a priori* that this method was adequate to identify CTL targets. Time and resources constraints limited the number of experiments performed but

sufficient progress was made to indicate that this expression technique has good potential for examining EAV CTL targets.

An interesting finding was the ease with which heterologous expression could be achieved using this technique. The generation of recombinant baculovirus required the construction of a 'chimeric' baculovirus shuttle vector containing a large fragment of the pcDNA3 / ORF7 recombinant plasmid. This laborious process was also used to generate a baculovirus expressing EAVORF5. However, some other experiments were performed to simplify the cloning strategy in order to generate recombinant baculoviruses for mammalian cell expression and promising results were obtained. A second 'chimeric' shuttle vector was constructed by cloning the A' tailed *NruI-SmaI* fragment of the pcD / ORF7 plasmid into the *BamHI* restriction site of pVL1393 instead of the *SmaI* site. This *BamHI* site was previously disrupted by filling the 5' overhangs of the digested plasmid using Klenow polymerase and T' tails added to the 3' ends. By doing this, the only *BamHI* restriction sites present in the baculovirus shuttle vector are those flanking the EAVORF7 allowing the quick insertion of any ORF containing *BamHI* restriction sites without the need to clone first the gene of interest in pcDNA3 and subclone the mammalian expression cassette into the pVL1393 plasmid.

The high levels of expression achieved in EDC make baculovirus transduction a potential effective vaccination technique for horses. Intramuscular injection of a recombinant baculovirus containing the pseudorabies gB gene under the control of the mammalian CAG promoter induced an antibody response in mice (Aoki *et*

al., 1999). However, during our studies, co-infection of cells with recombinant vaccinia MVA / T7 was necessary for expression of the recombinant gene. Further work is necessary to determine why N expression was inefficient in the absence of MVA / T7 co-infection.

Although it is still possible to use baculovirus transduction to determine CTL targets, is worth considering alternative approaches for expressing EAV genes in EDC. Vaccinia virus has been used extensively to express heterologous genes in mammalian cells and used in multitude of applications, including CTL assays. The use of low cytopathogenic vaccinia strains such as MVA or the use of other poxviruses such as fowl pox or canary pox is much preferred for generating targets for CTL assays. Some recombinant vaccinia viruses encoding EAV ORF's have been made using the WR strain and can be made available by Professor Rottier to test whether these can be applied in the CTL assay. Other suitable expression systems include retrovirus expression vectors which have been used successfully to map CTL targets of equine infectious anemia (Lonning *et al.*, 1999) or alphavirus expression systems such as those based on Sindbis, Semliki Forest or Venezuelan Equine Encephalitis viruses (Bennet, 1999; Hahn *et al.*, 1992; Balasuriya *et al.*, 2000).

Identification of the viral gene products that provide CTL targets will provide additional information about virus-host interactions during EAV infection in the horse. One goal would be the induction of protective immunity after *in vivo* expression of CTL epitopes using DNA or live virus vaccination. This would provide valuable information on the relative contribution of cellular immunity to

protection against infection. Also, incorporation of these proteins in marker vaccines may increase the efficacy of such vaccination approaches. As alluded to earlier, identification of CTL epitopes would help to elucidate whether genetic mutations generated during infection represent an immune evasion mechanism of EAV.

For complete interpretation of data generated from CTL epitope mapping experiments it is essential to know the MHC-I background of the animals from which effectors and targets have been obtained. This has been one of the major difficulties encountered during the course of these studies. MHC-I typing in horses has been performed by other workers using specific equine sera obtained from primiparous pregnant mares (since these develop antibodies against the paternal MHC-I antigens carried by the conceptus). These reagents are scarce and at the time the experiments were performed it was not possible to obtain them. It is essential that for this work to progress efficiently in the future, effectors and target cells must be derived from animals of known MHC-I haplotype.

In this research project two novel approaches have been used for the generation of marker vaccines against EAV and methods to evaluate cytotoxic T lymphocyte responses developed. The essential findings of the thesis provide new and interesting information about EAV vaccinology/immunology that now provide the basis for rational approaches to be undertaken to disease prevention.

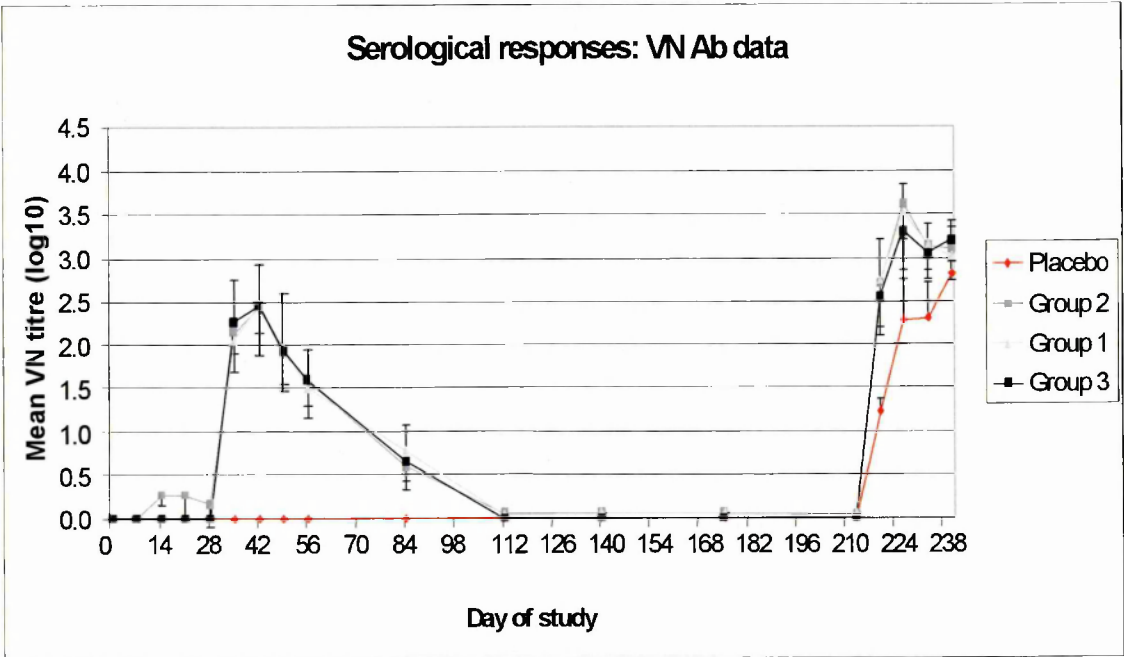


Figure 6.1. Serological responses following 6hisG₁ecto vaccination and subsequent to challenge. Vaccine doses were 0 μ g (placebo), 4 μ g (group 1), 8 μ g (group 2) and 32 μ g (group 3).

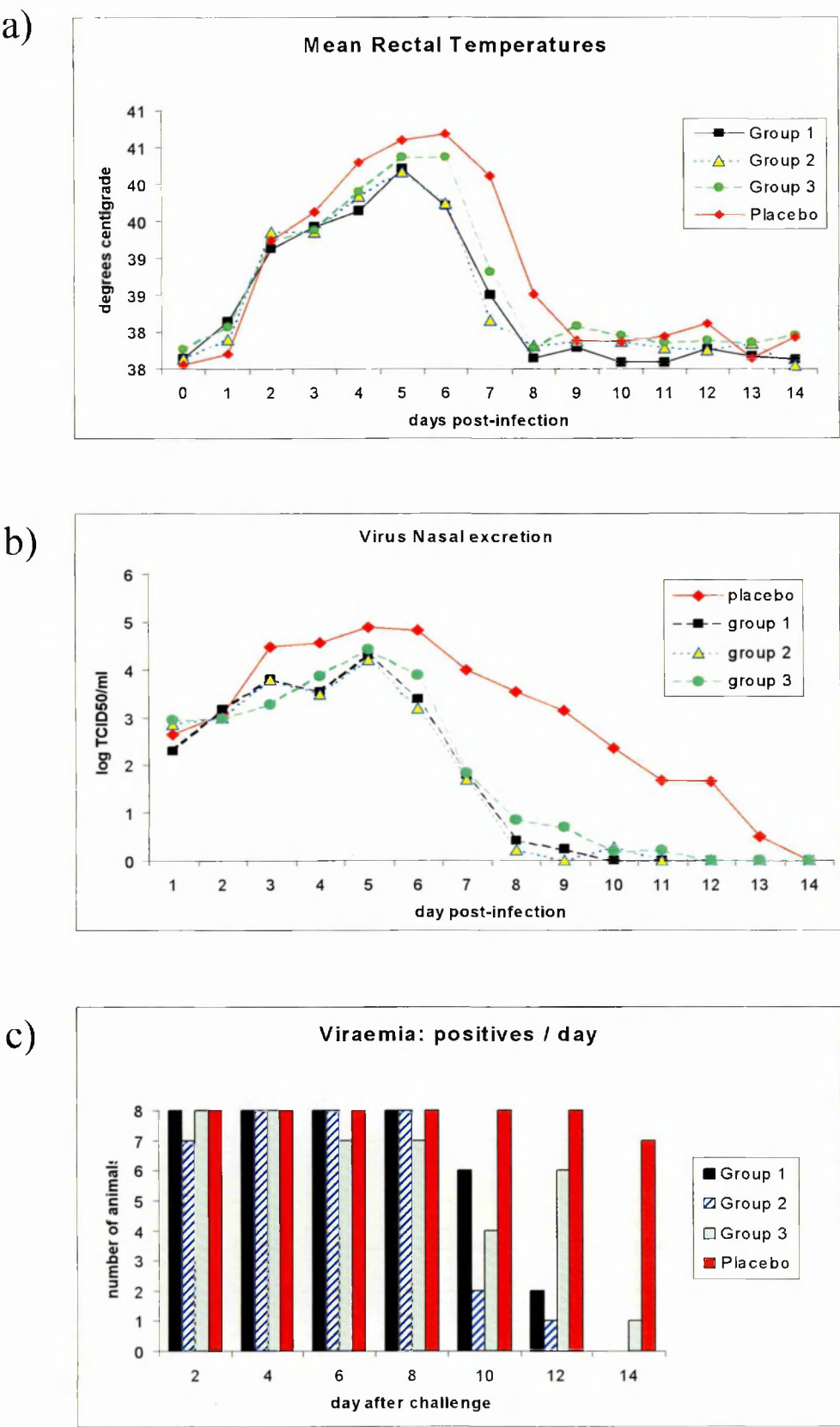


Figure 6.2. Responses of 3 groups of ponies vaccinated with 4 μ g (Group 1), 8 μ g (Group 2) or 32 μ g (Group 3) of 6hisG_Lecto vaccine and a pony group injected with a placebo to EAVLP3A+ challenge. Mean rectal temperatures (a), mean virus titres from nasopharyngeal secretions (b) and numbers of ponies presenting viraemia per day (c) are shown.

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